Críticos con los críticos del SIDA

Apuntes en torno al libro “El Rapto de Higea”

Como lectores habituales que éramos de la editorial *Virus* y también como trabajadores sanitarios, recibimos con gran interés la noticia de la publicación de un nuevo texto crítico centrado en el ámbito de la "salud y la enfermedad". El libro *"El Rapto de Higea: mecanismos de poder en el terreno de la salud y la enfermedad"* (ERH en los sucesivo) escrito por Jesús García Blanca y publicado por *Virus* a finales de noviembre del 2009 incluye un capítulo específico dedicado a la infección por el Virus de la Inmunodeficiencia Humana (VIH) (*Capítulo 4 Caso SIDA como ejemplo: los límites de la rebeldía*). Tras la lectura de dicho capítulo decidimos realizar un dossier que recogiera los diferentes errores que detectamos en los datos y citas en los que el autor basaba sus argumentaciones y remitirlo al colectivo editorial. Nos parecía importante que, dadas las implicaciones que esta infección puede suponer para la salud individual y colectiva, los datos y citas incluidos fueran extremadamente rigurosos. El colectivo editorial, tras evaluar durante algunos meses el dossier, nos comunicó que había decidido seguir adelante con la reedición del texto en su formato original, pese a los múltiples fallos puestos de manifiesto. Desde *Virus* se pidió que el dossier fuera difundido públicamente y nos pusimos manos a la obra: adaptamos el texto intentando facilitar su lectura y se incluyeron un par de nuevos puntos que por falta de tiempo no se analizaron inicialmente.

Hemos aportado las citas originales de aquellos datos recogidos en el texto y se ha incluido un anexo con los artículos más relevantes para que puedan ser consultados directamente. Cada cual que saque sus propias conclusiones.

Antes de entrar en materia y para que los lectores menos familiarizados con el tema puedan ubicarse y comprender algo mejor ciertos conceptos que utilizaremos en el texto, intentaremos explicar en pocas líneas en qué consiste la infección por el VIH. Sabemos que es imposible recoger en unos poco párrafos lo que extensos tratados de medicina y miles de artículos científicos no consiguen explicar de forma completa, así que pedimos perdón por adelantado por todos los errores que el “resumen” pueda conllevar.

Mapa de Navegación por el Dossier

1. Anotaciones sobre el Virus de la Inmunodeficiencia Humana

El VIH es un retrovirus formado por tres estructuras: 1) el genoma del virus, constituido por dos hebras de ARN unidas a las enzimas de la replicación, 2) una cápside, y 3) una envoltura lipídica. Esta última procede de la célula huésped y en ella se insertan las proteínas que facilitan la unión entre el virus y los receptores CCR5 y CXCR4 de las células inmunes, fundamentalmente linfocitos tipo CD4, así como macrófagos y células dendríticas. Gracias a esta unión el virus entra en dichas células y las infecta. Dentro de la célula infectada el virus se replica a través de la enzima viral "transcriptasa inversa", dando lugar a una cadena de ADN a partir del propio ARN del virus. Este nuevo ADN viral se integra en el ADN celular mediante la enzima "integrasa", la cual permite la síntesis de las proteínas y otros elementos que formarán los nuevos virus (1).

Los linfocitos CD4 infectados pueden permanecer en estado latente, manteniendo una replicación constante del VIH durante años, o pueden destruirse como consecuencia de la replicación intracelular masiva del VIH. Este último fenómeno conlleva el descenso del recuento de células CD4, y el consiguiente estado de inmunodepresión. Muchos estudios han demostrado que existen otros mecanismos que participan en la progresiva inmunodepresión de estos sujetos, como el freno en el desarrollo de nuevos linfocitos CD4, o el aumento de la apoptosis (muerte celular programada) en presencia del VIH(1).Como consecuencia de estos fenómenos, la enfermedad producida por el VIH tiene lugar en tres fases: una primera fase aguda tras 2-4 semanas del contagio, una fase crónica que en puede durar años y una fase final caracterizada por el descenso progresivo de los CD4 y la aparición de las infecciones oportunistas, alcanzando en última instancia el estadio SIDA (Síndrome de Inmunodeficiencia Humana Adquirida)(1).

2. ¿Un montaje?

Desde la introducción del texto el autor expone la conclusión que ha alcanzado en estos años y que decide denominar como *"Montaje VIH/SIDA": "El VIH/SIDA es una construcción planificada conscientemente con consecuencias criminales directas – la gente afectada por el montaje en sí - e indirectas - la gente engañada y afectada por problemas que el montaje impide abordar adecuadamente"* (ERH pág. 227)". Según el autor este montaje *"actúa a múltiples niveles"* como parte de la *"estrategia global de control imperial"* y está conformado por los "*científicos sin escrúpulos, el poder económico y mediático de las multinacionales... ONG, Asociaciones Profesionales, Universidades, Centros de Investigación… que están provocando un silencioso genocidio"* (ERH pág. 227).

En resumen, los cientos de miles de personas que se dedican de forma directa o indirecta al estudio de la infección por el VIH, investigación de fármacos y desarrollo de vacunas y aquellos que tratan y cuidan a las personas infectadas por el VIH están participando activamente en un *"montaje"* que ocasiona este *"brutal genocidio"*. Iremos revisando a lo largo del texto los datos que aporta (o no) el autor para sustentar esta controvertida afirmación.

Lo primero que queremos puntualizar es el error que el autor comete al equiparar los conceptos de "*infección por VIH" y "SIDA"* refiriéndose a una única entidad, *"Montaje VIH/SIDA"*. Actualmente la inmensa mayoría de las personas infectadas por el VIH que reciben un tratamiento correcto no progresan a estadio SIDA. Cientos de estudios han demostrado una disminución de la incidencia de las infecciones oportunistas características del estadio SIDA así como de la mortalidad desde que en 1997 empezaron a utilizarse los tratamientos combinados (Terapia Anti-Retroviral de Gran Actividad o TARGA, en Inglés HAART). La gráfica inferior (Figura 1) corresponde a un estudio realizado en un extenso grupo de infectados por el virus (2), y en ella se observa el descenso de las infecciones oportunistas (citomegalovirus (CMV), *Mycobacterium avium complex* (MAC), de la neumonía por *Pneumocystis* (PCP) y la esofagitis por *Candida)* desde el inicio de estas terapias. En conjunto, los porcentajes de estas infecciones (por 1000 personas/año) han descendido comparando tres periodos de tiempo, 1994-1997 (89%), 1998-2002 (25%), 2003-2007 (13%), siendo este porcentaje aún menor entre las personas que se han infectado recientemente. Por todo esto, equiparar infección por VIH a SIDA resulta completamente distorsionado y estigmatizador.

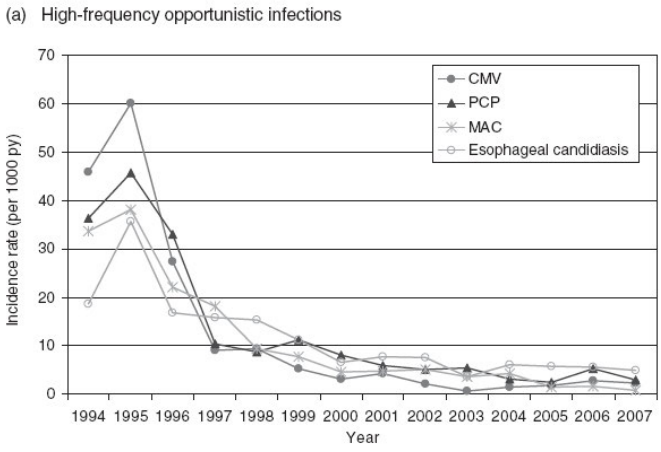


Figura 1. Tabla extraída Buchacz et al(2)

3. ¿Quiénes son los "críticos"?

Entre los "críticos" con las tesis oficialistas de la infección por el VIH, el autor diferencia varios grupos (ERH 238):

- Aquellos que *"consideran que el SIDA es una enfermedad"* pero cuestionan ciertos aspectos de las tesis oficiales desde una perspectiva "*científico-médica*" (ERH 238). En este primer grupo se encuentran los denominados "disidentes", entre los que destacan las críticas que en los años 80-90 plantearon a la comunidad científica personajes como P. Duesberg. Este virólogo alemán creía en la existencia del virus y en su aislamiento, pero centraba su discurso en la ausencia de relación entre el VIH y el SIDA. Para este grupo la verdadera causa del SIDA eran determinados factores relacionados con la forma de vida, como el consumo de drogas. Sus teorías tuvieron una amplia resonancia inicial y fueron publicadas a finales de los 80 en prestigiosas revistas científicas como *Science* o *The Lancet (*ver el debate *"HIV causes AIDS").* Tras varias décadas de investigación y experiencia clínica estas críticas han demostrado ser totalmente erróneas (han pasado más de 30 años y desde entonces algo ha llovido) como veremos más adelante. En una línea también científico-médica se encuentra Eleni Papadopulos-Eleopulos, a la que el autor otorga una gran credibilidad por sus "investigaciones críticas rigurosas" (ERH 238). El nombre de Eleni Papadopulos-Eleopulos se hizo conocido por su participación en la defensa judicial de André Parenzee, un hombre australiano que fue condenado por mantener relaciones sexuales sin protección durante años con 3 mujeres ocultando que estaba infectado por el VIH y contagiando el VIH a una de sus parejas (3).
- El otro grupo en el que el autor parece alinearse está conformado por aquellos que consideran que la infección por el VIH *"es un montaje"* utilizado como *"estrategia de control imperial"*.

4. ¿Nadie ha presentado pruebas del aislamiento del VIH? (ERH 243)

El autor plantea que para poder hablar de aislamiento de un nuevo virus deben cumplirse unos criterios desarrollados a partir de los clásicos "Postulados de Koch". Robert Koch (1843-1910) fue un bacteriólogo alemán que definió hace más de un siglo los principios que deben cumplir las enfermedades infecciosas para establecer la relación causal entre el agente infeccioso y dicha enfermedad. Estos postulados se resumen en (4):

1.- Un microorganismo específico puede encontrarse siempre asociado a una enfermedad determinada.

2.- El microorganismo puede aislarse y cultivarse en un cultivo puro en el laboratorio.

3.- El cultivo puro del microorganismo produce la enfermedad al ser inyectado en un organismo sano.

4.- Es posible recuperar el microorganismo a partir del animal infectado.

Uno de los puntos clave que el autor refiere como fundamental en su teoría es que "*con el VIH no se han cumplido hasta ahora"* estos postulados*; "nadie ha publicado jamás micrógrafos del «VIH»; nadie ha podido obtener ejemplares aislados del «VIH» para poder establecer cuáles son sus proteínas o su información genética; nunca se han realizado experimentos de control; y por supuesto, nadie ha logrado jamás repetir lo que hizo Gallo en 1984*" (ERH 243).

Ante un alegato de semejante rotundidad y trascendencia, el colectivo editorial, a nuestro entender, debería haber contrastado estas afirmaciones. Una breve búsqueda en bases de datos científicas como Pubmed demuestra que el aislamiento del VIH está perfectamente constatado desde hace años y que existen miles de microfotografías que así lo atestiguan (5, 6, 7; Fig 2, 3, 4), además de las que aportó R. Gallo hace 30 años, y que el autor de ERH considera *"fraudulentas"*. Asimismo la secuenciación genética del virus y sus proteínas también están bien descritas (8; Fig 5). Por muchas fotografías o documentación que aportemos, el autor probablemente mantendrá que se trata de un montaje y las denominará *"micro vesículas"*, productos de *"excreción celular"* o cualquier otro término, ya que el reconocimiento de la existencia del virus desmonta completamente sus teorías. Sin embargo este hecho es tan evidente que ha sido incluso reconocido por otros críticos con un discurso más elaborado, como es el caso de P. Duesberg.

Respecto al resto de Postulados de Koch, desde que se conocieron los primeros afectados por la infección se han comunicando casos de personal de laboratorio y trabajadores sanitarios infectados accidentalmente mientras manejaban con el virus, que tras el contagio han presentado enfermedades incluidas dentro del estadio SIDA, sin presentar ninguno de los factores de riesgo que el autor denomina "estresantes", como los *"poppers"* (página 282 ERH) la adicción a drogas inyectables o la *"hemofilia"*(9, 10). Es decir, que trabajadores de laboratorio, previamente sanos, tras un contacto con el virus aislado presentan con el paso de los años infecciones oportunistas características del estadio SIDA, y el virus puede ser recuperado de la sangre de estas personas infectadas, cumpliéndose así todos los criterios de Koch de los que tanto habla el autor. Por otro lado también se ha podido demostrar el desarrollo de SIDA en animales a los que se ha inoculado el virus (11).

Fig. 2 Organización de las partículas inmaduras y maduras del VIH. Imágenes de formas inmaduras (d) y maduras (e) del VIH obtenidas por crioelectromicroscopía electrónica (5).

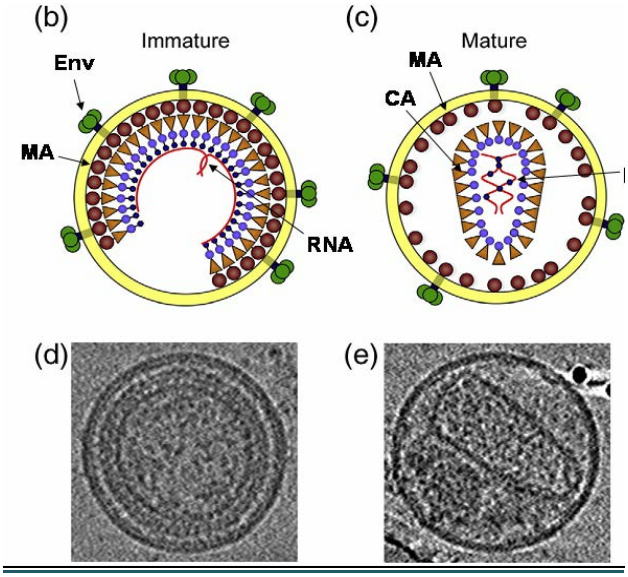


Fig 3. Fotografía del virus VIH al microscopio electrónico (izquierda) y esquema simplificado de su estructura (derecha). Se puede observar la cápside de color verde en el esquema (6).

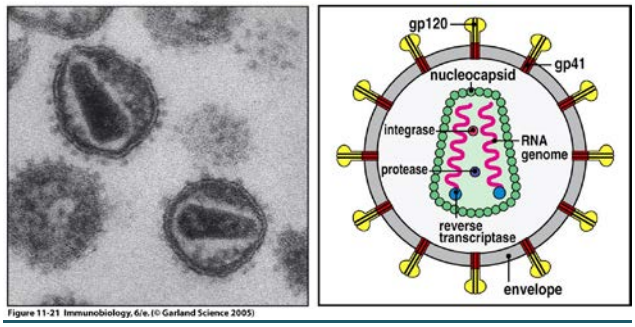


Fig 4. Observación mediante criomicroscopía electrónica de cápsides del virus VIH. En A se observan viriones maduros de VIH purificados desde linfocitos T previamente infectados. Escala: 150 nm. En B se muestra un grupo de cápsides de VIH aisladas. Pueden verse las típicas formas cónicas. Escala 100 nm. C muestra un par de cápsides aisladas. Escala 50 nm (7).

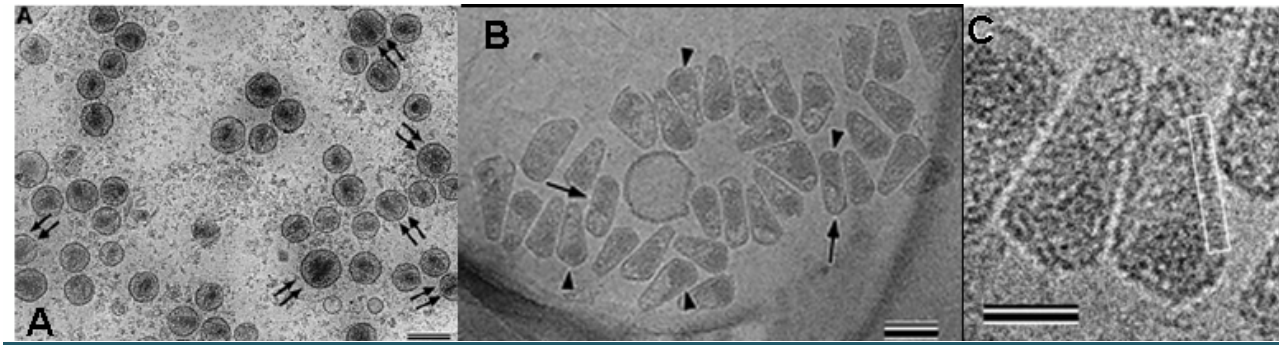
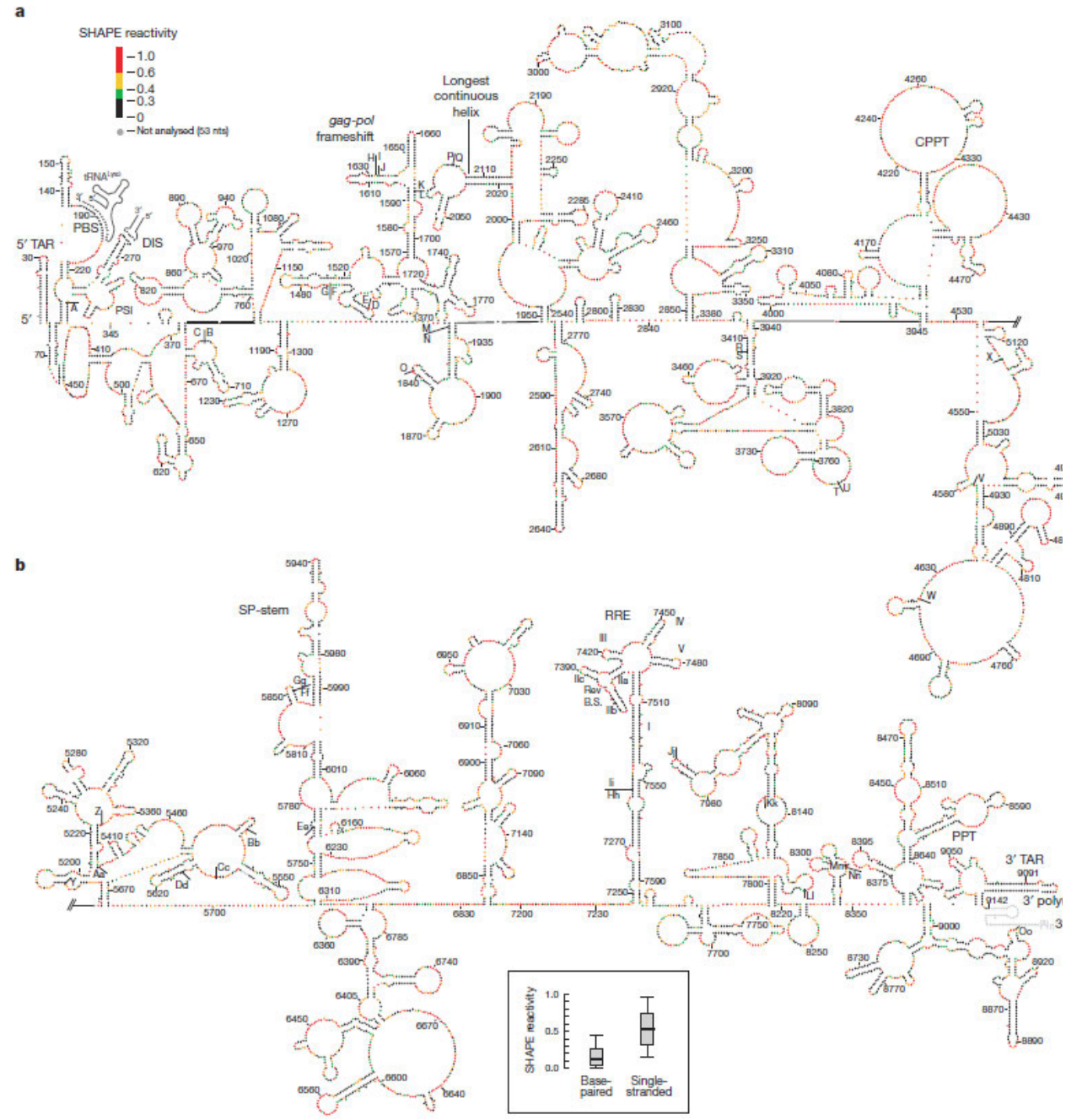


Fig 5. Estructura del genoma del VIH. Se muestran los extremos 5´(a) y 3´(b) (8).


a
SHAPE reactivity
1.0
0.6
0.4
0.3
0
Not analysed (53 nts)
5′ TAR
PBS
DIS
PSI
gag-pol frameshift
Longest continuous helix
CPPT
b
SP-stem
RRE
PPT
3′ TAR
3′ poly
SHAPE reactivity
1.0
0.5
0.0
Base-paired
Single-stranded

5. Causa del SIDA

Entonces ¿cuál es la causa del SIDA? Para el autor lo que verdaderamente subyace al SIDA son "*las agresiones psicológicas, traumáticas, infecciosas nutricionales y tóxicas*", que "*provocan un desequilibrio importante en el organismo, una situación de estrés"* (285 ERH). También dice que *"es un montaje y por tanto no una enfermedad a tratar, ni siquiera por métodos alternativos".* Sin embargo en la tabla de la página 287 se contradice, atreviéndose a proponer un *"tratamiento bio-regenerativo para los estados de enfermedad oficialmente considerados SIDA"*. Este supuesto tratamiento consiste en masticar bien la comida, tomar glutatión, antioxidantes, agaragar y cartílago, e incluso, *"en algunos casos, **resignarse a morir**"* (ERH 289), aunque no aporta ningún estudio aleatorizado que haya demostrado la eficacia de estos tratamientos tan particulares. Desde luego que *"resignarse a morir"* no parece un mensaje muy alentador para aquellos que estén infectados por el VIH ni para sus terapeutas, especialmente teniendo en cuenta que actualmente es una infección tratable con menor mortalidad que otros muchos problemas crónicos.

La relación causal entre HIV y SIDA se encuentra plenamente demostrada en múltiples estudios. A modo de ejemplo destacaremos algunos:

1. Un estudio describe la evolución durante 10 años de 11 niños recién nacidos que de forma accidental habían recibido una transfusión de sangre de un mismo donante infectado por el VIH. De ellos, tres niños fallecieron a los 2.5 años, 5 entre los 6,2 y 11 años, y 3 permanecían vivos en el momento del estudio. Todos los niños presentaron una disminución progresiva de las defensas (CD4)(12).

2. Otro estudio siguió durante 9 años a 715 hombres que practicaban sexo con hombres (HSH). De ellos, 237 tenían anticuerpos frente al VIH desde el comienzo del estudio, 128 eran seronegativos pero durante el periodo de seguimiento se infectaron y positivizaron, y otros 350 se mantuvieron seronegativos todo el tiempo. El objetivo del estudio era determinar el número de infecciones oportunistas y los factores de riesgo de cada uno de los grupos. Se presentaron 136 episodios de enfermedades incluidas dentro del estadio SIDA, todas ellas en individuos infectados por el VIH. No hubo ni un sólo caso definitorio de SIDA entre las personas no infectadas pesar de tener estilos de vida similares. Se analizaron los factores de riesgo que según los críticos como Duesberg eran la causa real del SIDA, como los nitritos inhalados (*poppers*) y otras drogas, siendo la prevalencia de su uso similar entre ambos grupos. El descenso de las células defensivas (CD4) era independiente del uso de estas drogas y el único factor de riesgo que se relacionaba con

el descenso de los CD4 y presencia de eventos SIDA estar infectado por el VIH. En la figura 6 se puede apreciar como el descenso de los CD4 no se relaciona con el uso de nitritos inhalantes(13).

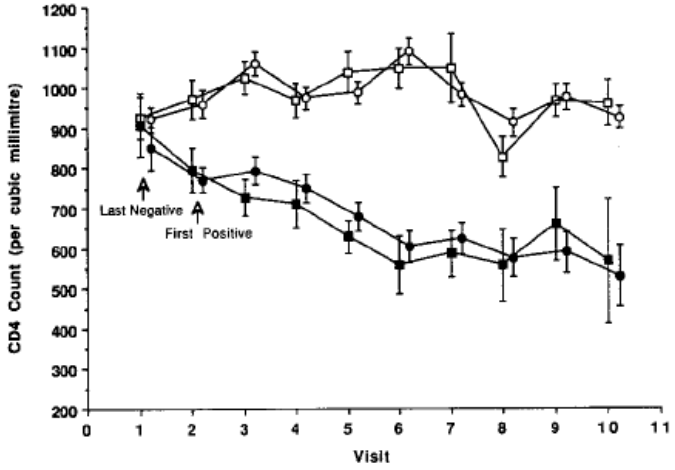


Figura 6: Recuentos de CD4. En negro aquellos previamente negativos frente al VIH que durante el seguimiento han virado a positivo (se han infectado) y en blanco los pacientes seronegativos durante el seguimiento. En ambos grupos se recogen los que han usado (redondel) y los que nunca han usado (cuadrado) nitritos inhalados. Se puede ver que no existen diferencias en el recuento de CD4 entre los que usan y no usan poppers, pero si entre los no infectados (seronegativos) y los que se han infectado, que presentan un descenso progresivo de CD4 tras la infección, a pesar de que partían de niveles similares cuando no estaban infectados(8).

Este estudio fue replicado por Duesberg en la revista *Lancet* argumentando que la administración de Zidovudina (AZT) podía ser la causa del descenso de los CD4 que experimentaban los pacientes infectados con el VIH (14). Los propios autores del estudio contestan posteriormente a Duesberg que han seguido a 19 pacientes VIH positivos que no habían utilizado drogas recreativas y que no han recibido Zidovudina, y se valoró su evolución durante el periodo previo al uso del AZT (antes de 1986), con un descenso medio de los CD4 de 138/uL al año. Los autores aseguran que estos pacientes no habían podido recibir AZT de ningún modo dado que aún no se distribuía en su área y eran el único hospital autorizado para su uso en los años sucesivos(15), descartándose así la hipótesis farmacológica, tanto para las drogas como para el AZT.

3. Antes de la aparición del VIH las alteraciones que aparecen asociadas al estadio SIDA como la neumonía por *Pneumocystis jirovecii* (PCJ), el Sarcoma de Kaposi (cáncer que aparece en pacientes con infección por el VIH en estadio SIDA) o la infección diseminada por *Mycobacterium avium complex* (MAC) eran extremadamente raras. Hasta 1967, solo se habían descrito 107 casos de neumonía por PCJ en EEUU, todos ellos en pacientes en situación de inmunodepresión severa (16). A finales de 1994, el CDC comunica 127,626 casos confirmados de neumonía por PCJ entre pacientes con SIDA en EEUU, muchos de ellos producidos antes de recibir tratamiento con AZT(17). Antes de confirmarse los primeros casos de SIDA, la incidencia anual del Sarcoma de Kaposi era sólo de 0.2 a 0.6 casos por millón de habitantes (18), y únicamente 32 personas en EEUU habían tenido una infección diseminada por MAC(19). En

1994, 36,693 casos confirmados de Sarcoma de Kaposi y 28,954 de MAC se habían producido entre los pacientes con infección por VIH en EEUU coincidiendo con la comunicación de los primeros casos de SIDA (17,19-21).

Estos problemas característicos de las personas con infección por el VIH que alcanzan un estadio SIDA comenzaron a presentarse de manera exponencial desde el inicio de los primeros casos de la infección y muchos de ellos aparecieron antes del uso de antirretrovirales como el AZT.

6. Infección infantil

Bajo el sensacionalista epígrafe de la *"máquina infanticida"* el autor del texto expone sus opiniones sobre el VIH infantil y la transmisión del VIH la madre al hijo durante el embarazo, parto y periodo neonatal. Cuando aparecieron los primeros casos de SIDA en los años 80, la transmisión vertical al niño ocurría aproximadamente en un 20% de las embarazadas seropositivas. Actualmente la transmisión vertical es infrecuente en España (1-2%), lo que desgraciadamente no ocurre en países con menos recursos.

6.1- Evidencia de transmisión madre- hijo del VIH durante el embarazo.

Según el autor no existe evidencia de que la infección se transmita durante el embarazo, parto o puerperio (ERH 303). Desde el inicio de los primeros casos de SIDA, se comprobó que existía una transmisión desde la madre al niño (22), tanto durante el embarazo como durante el parto y la lactancia, que ha sido comprobada en cientos de estudios posteriores.

Según el autor, ni el AZT ni la Nevirapina previenen la transmisión del VIH (ERH 305). Esta afirmación es absolutamente falsa y se puede comprobar fácilmente examinando el porcentaje de niños infectados antes de la utilización de la Zidovudina (AZT) y después. Ponemos por ejemplo un estudio realizado en Cataluña donde se aprecia un franco descenso de un 20,4% en el periodo 1987-1993 a un 3,5 % en el periodo 1997-2003 con el uso del AZT y posteriormente de la triple terapia (TARGA O HAART en inglés) durante el embarazo (Fig. 7 y 8)(23).

Figura 7

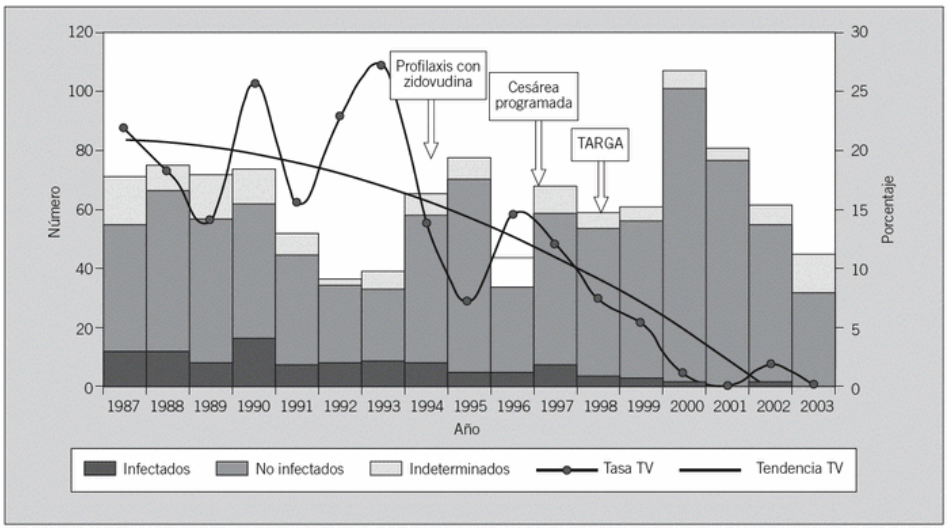


Figura 8

Transmisión vertical del VIH y características maternas y de las gestaciones por período de estudio

Característica	Período									p
	1987-1993			1994-1996			1997-2003			
	n[a]	%	IC del 95%	n[a]	%	IC del 95%	n[a]	%	IC del 95%	
N.º de niños	420			186			499			
Estado de infección										
Infectado	72	17,1	13,7-21,1	18	9,7	5,8-14,9	16	3,2	1,8-5,1	
No infectado	281	66,9	66,2-71,4	144	77,4	70,7-83,2	442	88,6	85,4-91,2	
Indeterminado	67	15,9	12,6-19,8	24	12,9	8,4-18,6	41	8,2	5,9-11,0	< 0,001
Tasa de TV	353	20,4	16,3-25,0	162	11,1	6,7-17,0	458	3,5	2,0-5,6	< 0,001
Edad gestacional										
< 37 semanas	73	18,3	14,7-22,5	34	20,0	14,3-26,8	121	25,2	21,4-29,3	
≥ 37 semanas	325	81,7	77,5-85,3	136	80,0	73,2-85,7	359	74,8	70,7-78,6	0,013
Peso al nacer										
< 2.500 g	120	29,3	24,9-33,9	46	26,0	19,7-33,1	116	23,5	19,9-27,5	
≥ 2.500 g	290	70,7	66,1-75,1	131	74,0	66,9-80,3	377	76,5	72,5-80,1	0,051
Tipo de parto										
Vaginal no instrumentado	121	30,2	25,7-34,9	80	45,4	37,9-53,1	110	23,5	19,7-27,6	
Vaginal instrumentado	38	9,5	6,8-12,8	16	9,1	5,3-14,3	26	5,5	3,7-8,0	
Cesárea programada	129	32,2	27,6-37,0	61	34,7	27,7-42,2	273	58,2	53,6-62,7	
Cesárea en el curso del parto	113	28,2	23,9-32,9	19	10,8	6,6-16,3	60	12,8	9,9-16,1	< 0,001
Vía de infección de la madre[b]										
UDVP	332	79,2	75,0-83,0	113	60,7	53,3-67,8	214	43,5	39,1-48,0	
Relaciones heterosexuales	72	17,2	13,7-21,1	60	32,3	25,6-39,5	250	58,8	46,3-55,3	
Desconocido	15	3,5	2,0-5,9	13	7,0	3,77- 11,6	28	5,7	3,8-8,1	< 0,001
Tiempo diagnóstico de infección en la madre										
< 1 año	93	58,5	50,4-66,2	42	49,4	38,4-60,5	75	17,9	14,3-21,9	
1-5 años	60	37,7	30,2-45,7	25	29,4	20,0-40,3	121	28,9	24,6-33,5	
> 5 años	6	3,7	1,4-8,0	18	21,2	13,1-31,4	223	53,2	48,3-58,1	< 0,001
	n	Mediana	RI	n	Mediana	RI	n	Mediana	RI	
Edad gestacional (semanas)	398	39,3	37,3-40,3	170	39,3	37,3-40,3	480	38	36,9-38,9	< 0,001
Peso al nacer (g)	410	2.842,5	2.400-3.152	177	2.880	2.445-3.280	493	2.820	2.520-3.150	0,961
Edad de la madre (años)	392	24,6	22,5-28	116	27,4	23,5-29,6	474	30,5	27,1-34,0	< 0,001
Tiempo de diagnóstico de infección (años)	159	0,5	0,07-2	85	1,3	0,0-4,5	419	5,8	2,3-10,3	< 0,001

[a]En algunas características, las categorías no suman el total de casos por no disponerse de información o por pérdidas;
[b]No aparecen las categorías de transmisión parenteral no-UDVP (5 casos) y transmisión perinatal (3 casos).
IC: intervalo de confianza; RI: rango intercuartílico; TV: transmisión vertical; UDVP: usuarios de drogas por vía parenteral.

Actualmente el porcentaje de transmisión vertical en nuestro medio es del 1,42%, como demuestra un estudio realizado en gestantes de la Comunidad de Madrid(24). En este estudio sólo se infectaron 9 recién nacidos de entre 635 madres VIH positivas en el periodo del trabajo (mayo 2000-diciembre del 2005). Sólo una de las 9 madres que transmitieron la infección al niño había recibido el tratamiento correcto durante el embarazo y el parto. De los recién nacidos infectados, 2 presentaron una neumonía por *PCJ* y uno de ellos falleció al mes de vida. Estos porcentajes son similares a lo que se ha descrito en otros países occidentales en donde la tasa de transmisión vertical ha disminuido hasta el 0,5-1% con la utilización de los antirretrovirales.

Por tanto la transmisión del VIH puede evitarse eficazmente recibiendo medicación antirretroviral en el embarazo y el parto, por lo que lanzar afirmaciones que pueden llevar a que una mujer gestante decida no tomar la medicación implica un riesgo directo para su hijo. Nos llama la atención la tranquilidad con la que el autor y aquellos que publican estas opiniones (no sustentadas en ningún dato), que abordan un tema que trasciende la decisión estrictamente personal de un adulto y afecta directamente a un niño que no ha podido decidir si quiere o no vivir infectado por el VIH.

6.2 Cesárea

Este punto es uno de los más sorprendentes del texto. El autor da a entender que las Recomendaciones del Plan Nacional del SIDA (PNS) indican que se debe hacer cesárea a todas las madres VIH positivas (ERH 304). Acto seguido cita a la Dra. Olza hablando sobre los problemas que acarrea la cesárea.

No entendemos por qué no ha explicado lo que realmente indican las recomendaciones del PNS sobre la cesárea, citando sólo aquellos fragmentos del texto que apoyan sus hipótesis (25). El documento recomienda que todas aquellas mujeres que han recibido tratamiento correcto durante el embazo tengan un parto por vía vaginal y sólo se recomienda la cesárea si la “carga viral es mayor de 1000 copias”, que actualmente supone un mínimo porcentaje de casos. La cesárea puede ser valorada en aquellos casos con carga viral detectable pero menor de 1000 copias, pero en ningún lugar se establece que una madre que haya tomado el tratamiento y se encuentre con una carga viral indetectable deba dar a luz mediante cesárea.

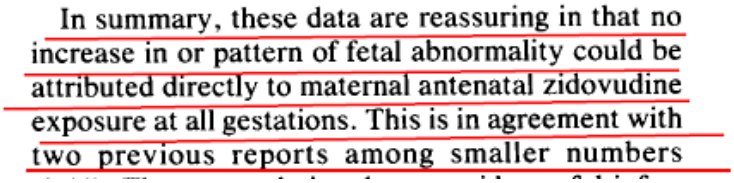
Las guías clínicas americanas son todavía más partidarias a limitar el uso de la cesárea en las gestantes VIH positivas e indican que no ha demostrado eficacia en las aquellas madres bien controladas durante el embarazo (26).

Estas recomendaciones son extensamente conocidas por todos aquellos agentes de salud que tengan un mínimo contacto con mujeres embarazadas VIH positivas y no entendemos el sentido de distorsionar lo que recogen las guías clínicas de referencia. Compartimos la preocupación que muestra el autor por reducir el número de cesáreas innecesarias que se realizan (sean madres VIH positivas o no) y la mejor estrategia para evitar una cesárea en el caso de las gestantes VIH positivas pasa por mantener un buen control virológico tomando correctamente el tratamiento.

6.3 Exposición a antirretrovirales durante la gestación

En la página 306 (ERH) el autor cita un artículo (27) de 1994 que, según refiere, pone de manifiesto que el AZT es teratogénico (capacidad de producir malformaciones si se usa durante el embarazo). Vamos a detenernos unas breves líneas en este trabajo, con un diseño bastante cuestionable (hasta los propios autores reconocen sus grandes limitaciones en la discusión) y con un limitado número de embarazadas (n=104). Llama la atención que en las propias conclusiones del trabajo citado se especifica claramente que no se ha podido establecer una relación entre el uso de AZT y las malformaciones que describe el autor (ver abajo las conclusiones recortadas directamente del propio artículo; Figura 9)

Figura 9


In summary, these data are reassuring in that no increase in or pattern of fetal abnormality could be attributed directly to maternal antenatal zidovudine exposure at all gestations. This is in agreement with two previous reports among smaller numbers

No sólo eso sino que varios de los niños que nacieron con alguna de las alteraciones descritas estaban infectados por el VIH o presentaban enfermedades de transmisión vertical como la toxoplasmosis congénita (más frecuente entre los hijos de madre VIH positivas con mal control de la infección) que pueden ocasionar las lesiones que el autor ha considerado causadas por el AZT, como era el caso de las calcificaciones cerebrales del niño con toxoplasmosis. Los propios autores del trabajo reconocen que el porcentaje de malformaciones es similar al obtenido en otros trabajos prospectivos realizados en niños sanos no expuestos al AZT.

En pocas palabras, el autor, citando este artículo, se inventa unas conclusiones contrarias a las expuestas por los propios autores del trabajo. Se inventa también que el trabajo es un “Ensayo Clínico”, cuando es fácil percatarse de

que no es así con solo mirar el resumen. Las únicas posibilidades que podemos suponer es que o no ha leído bien el trabajo o bien lo ha copiado directamente de otra persona que tampoco se ha leído el artículo. Llama la atención que se cite un trabajo realizado en la India en 1994 con un escaso número de gestantes en un libro de reciente publicación, existiendo una gran cantidad de trabajos actuales con un número de embarazadas inmensamente superior y de mejor calidad en cuanto al diseño.

Existen múltiples trabajos de bien diseñados que buscan establecer la seguridad de los antirretrovirales durante el embarazo. Un estudio recientemente publicado con 2202 niños expuestos a antirretrovirales (número "ligeramente" superior al del anterior artículo) (28) no ha encontrado relación entre la utilización del AZT y la presencia de malformaciones en los recién nacidos. Sin embargo existen otros antivirales como el Efavirenz (entre otros antirretrovirales) parecen mostrar una mayor tasa de malformaciones y actualmente no están recomendados durante la gestación. Las propias guías clínicas recogen los riesgos potenciales y recomiendan o desaconsejan el uso de cada uno de estos fármacos durante la gestación en función de las evidencias encontradas. No entendemos que se intente lanzar un mensaje alarmista cuando existen importantes estudios sobre el uso de antirretrovirales durante el embarazo con información accesible y totalmente pública sobre la seguridad (o no) de cada uno de los distintos fármacos.

6.4 Encefalopatía por VIH

La encefalopatía VIH es un problema dramático que aparece con 30 veces más frecuencia en los niños infectados por transmisión vertical que en los adultos y está ocasionado por daño directo del VIH a nivel cerebral, especialmente sensible durante el desarrollo intraútero y en los primeros meses de vida (29). Cursa con retraso del desarrollo, disminución del crecimiento cerebral y problemas motores muy graves, que en muchos casos condenan a estos chicos a una silla de ruedas. Desde la utilización del TARGA se ha producido un marcado descenso de los casos, siendo actualmente infrecuente en niños de nuestro medio que reciben tratamiento desde el nacimiento. En un estudio realizado en la Comunidad de Madrid publicado recientemente se puede apreciar un descenso marcado de la encefalopatía pasando de un 4,2% en el periodo de 1990-1996 a un 0,54% del 2000-2006 (30) (Fig.12 abajo).

7. Diagnóstico

7.1 Recuento linfocitario

El tema del diagnóstico de la infección es otro de los grandes caballos de batalla del autor. En primer lugar la crítica que plantea en torno al contaje de los linfocitos (ERH 253) está absolutamente errada. El contaje se realiza con los mismos instrumentos técnicos (citómetros) que se utilizan en el diagnóstico de otras inmunodeficiencias primarias y secundarias. Pongamos un ejemplo sencillo: los denominados "niños burbuja" que tienen la enfermedad conocida como Inmunodeficiencia Combinada Severa, ligada a distintas alteraciones genéticas, quienes presentan un déficit muy grave de linfocitos y otras células defensivas desde el nacimiento. Estos niños sufren graves infecciones oportunistas (similares las que padecen los pacientes en estadio SIDA) si no se realiza un trasplante de médula ósea de forma precoz. En ellos el contaje de linfocitos se encuentra muy disminuido, utilizando exactamente las mismas técnicas que se emplean para el recuento en los pacientes VIH, y no aparecen las caprichosas oscilaciones que refiere el autor. Si está criticando las técnicas de recuento linfocitario y las equipara a una especie de *"lotería*" (ERH 254) entendemos que las miles de enfermedades en las que se emplea este proceso diagnóstico (Inmunodeficiencias primarias y secundarias, enfermedades autoinmunes, etc.) deben ser puestas en tela de juicio. Parece que el complot sigue creciendo hasta límites insospechados.

7.2 Test de anticuerpos

Los test de anticuerpos (o estudios serológicos) se emplean extensamente en medicina para el diagnóstico de cientos de enfermedades infecciosas: fiebre botonosa, brucelosis, dengue, mononucleosis infecciosa, hepatitis virales, sarampión, rubeola, varicela, infecciones del grupo herpes, sífilis, toxoplasmosis... De nuevo el autor nos asombra con un nuevo descubrimiento: la ausencia de cualquier valor de estos test de anticuerpos.

Todos los test de anticuerpos utilizados en las distintas enfermedades tienen una mayor o menor posibilidad de dar falsos positivos (pacientes sanos en los que el test ha sido positivo) y falsos negativos (enfermos que han dado negativo al test). Este hecho no es diferente en el VIH, y en todos los manuales aparece la potencia de los distintos tests (las denominadas "sensibilidad" y "especificidad" de las pruebas), tanto de los test de EIA (Enzimoinmunoanálisis), como Western-Blot, y se incluyen las causas de falsos positivos y negativos.

La sensiblidad de las pruebas de EIA que se utilizan como screening en el VIH es muy elevada situándose en torno al 99,5% (1). Las pruebas de Western Blot se utilizan en la confirmación serológica, y presentan una mayor especificidad (menor tasa de falsos positivos) que los test de EIA (ronda el 99%). Se puede obtener más información sobre las pruebas serológicas del VIH en la página de la Sociedad Española de Microbiología, sección de Diagnóstico del la infección por el VIH http://www.seimc.org/documentos/ protocolos/microbiología.

Los resultados falsamente positivos o negativos en las pruebas serológicas pueden aparecer (aunque son infrecuentes en el caso del VIH) al igual que en cualquier otra infección, pero este hecho no invalida su utilidad clínica y menos aún implica que no existan estos gérmenes. A menos de que la mononucleosis infecciosa, la sífilis y otros miles de infecciones formen también parte el *"montaje VIH/SIDA"*, hecho que parece bastante improbable.

7.3 Carga viral, PCR, Test de resistencias:

Actualmente se pueden utilizar estas técnicas de biología molecular para confirmar el diagnóstico en ciertos casos (ej: recién nacidos), valorar cuál está siendo la respuesta al tratamiento (carga viral) y estudiar el desarrollo de resistencias a fármacos antirretrovirales que pueden aparecer en el VIH. Este tipo de procedimientos tienen una elevadísima sensibilidad y especificidad en el diagnóstico, alcanzando límites de detección muy bajos (25-40 copias/ml), y han permitido un desarrollo exponencial del conocimiento de la infección por el VIH. Estas técnicas son ampliamente usadas en virología en el diagnóstico de cientos de enfermedades: CMV, hepatitis B y C, Virus de Epstein Barr, Adenovirus, Virus Herpes, Enterovirus, y un largo etc. Todos los virólogos aceptan su gran utilidad, con las limitaciones que toda técnica diagnóstica pueda tener.

En su argumentación crítica contra la técnica de la PCR, el autor cita un artículo de Coste, de 1997. Este artículo es una breve Carta al Director que evalúa 3 kits comerciales en la detección de 15 muestras de diferentes subtipos de VIH (hecho que parece haber olvidado el autor), los cuales figuran en la tabla original del estudio (Fig. 10). En el artículo original el subtipo aparece recogido en la columna de la izquierda con las letras A-H (la hemos marcado en rojo). Podemos ver como el autor (u otra persona) ha recortado esa columna en la tabla del libro para omitir una información esencial para entender el fin del estudio, que consiste en explicar que dada la variabilidad genética del VIH cada test está diseñado para identificar los subtipos más prevalentes en cada zona geográfica, por los que subtipos menos frecuentes

pueden dar resultados discordantes. Este hecho es ampliamente conocido y ha sido solventado en parte con las nuevas técnicas de PCR.

Fig. 10

Tabla del artículo original.

Subtype	HIV-1 strain	HIV-1 RNA copies per ml of HIV-seronegative plasma		
		RT-PCR[a]	bDNA	NASBA
A	DJ258	<400	111.500	100.000
A	DJ263	<400	79.800	60.000
B	SF2	225.500	38.000	240.000
B	III-B	54.000	17.000	360.000
C	ZAM18	78.300	70.000	66.000
C	ZAM20	178.800	125.800	420.000
D	UG270	179.800	29.200	170.000
D	UG274	320.000	41.400	32.300
E	CM241	18.800	72.800	35.000
E	CM235	4.700	52.000	15.000
F	163.3069	36.200	94.000	57.000
F	162.3070	2.800	78.100	26.000
G	G98	254.700	269.000	<400
G	LBV21	184.500	295.000	<400
H	VI557	950.000	587.000	125.000

[a] RT-PCR, coupled reverse transcription–polymerase chain reaction amplification process (Amplicor Monitor by Roche Diagnostics Systems, Neuilly, France); bDNA, branched DNA amplification process (Quantiplex HIV-1 RNA by Chiron Corporation, Cergy Pontoise, France); NASBA, nucleic acid sequence–based amplification procedure (HIV-1 RNA QT assay by Organon Teknika, Fresnes, France).

Tabla sin la primera columna. ERH

Comparativa entre mediciones de carga viral			
VIH-1	RT-PCR	bDNA	NASBA
DJ258	<400	111,500	100,000
DJ263	<400	79,800	60,000
SF2	225,500	38,000	240,000
III-B	54,000	17,000	360,000
ZAM18	78,300	70,000	66,000
ZAM20	178,800	125,800	420,000
UG270	179,800	29,200	170,000
UG274	320,000	41,400	32,300
CM241	18,800	72,800	35,000
CM235	4,700	52,000	15,000
163.3069	36,200	94,000	57,000
162.307	2,800	78,100	26,000
G98	254,700	269,000	<400
LBV21	184,500	295,000	<400
VI557	950,000	587,000	125,000

8. Tratamiento y pronóstico

El autor expone (273 ERH) que *"los hechos clínicos documentados han ido confirmando que los pacientes que no toman los antirretrovirales gozan de mejor salud"*. Para ello cita varios trabajos que según él confirman este hecho.

Un trabajo Publicado por Candotti en 1999 en el *Journal of Medical Virology* (31) (no en "Medical Medicine" como figura en la cita del libro), según el autor, revela que *"de los 68 no-progresores a largo plazo (más de 10 años) ninguno estaba en terapia antirretroviral"*. Revisando dicho artículo encontramos otra sorpresa: el estudio buscaba conocer las características de los lentos progresores a partir de parámetros virológicos como la carga viral. Para ello definen unas características que deben cumplir obligatoriamente los pacientes que se incluyen en el estudio. Una de ellas es... (¡¡¡atención!!!!) que nunca hayan recibido tratamiento antirretroviral previamente. Es por tanto normal que los 68 pacientes incluidos en este estudio no hayan recibido tratamiento antirretroviral previo pues es precisamente un

criterio para no incluirlos en el estudio. No encontramos por tanto ningún sentido a la cita y por supuesto no sustenta en modo alguno la afirmación del autor.

Otra de las citas que el autor expone es la de J. Levy del *Lancet* de 1998 (32). Este artículo es sólo una carta de opinión, no un estudio con datos propios. El Dr. Levy trata en esta carta sobre cuándo se debe empezar el tratamiento antirretroviral, pues en el año 98 aún no había grandes consensos sobre la triple terapia, ni sobre el momento en que debía iniciarse el tratamiento. En ningún momento compara la supervivencia de los no tratados con los tratados, por lo que este documento tampoco apoya las afirmaciones del autor.

Presenta una nueva cita de Hogervorst que el autor localiza en 1996, (pero que en realidad es del 1995, adjuntamos la cita real para que os sea más sencillo que a nosotros encontrar el artículo) (33), en la que, según el autor, se dice que *"ninguno de los enfermos asintomáticos a largo plazo recibió ningún medicamento antiviral durante el estudio"*. En este estudio comparaban las características de varios grupos de pacientes en Holanda. Escogieron tres grupos: uno de pacientes no progresores (más de 7 años infectados y asintomáticos) y otros dos grupos de progresores (lentos y rápidos) y compararon sus características de carga viral, inmunología, etc. La conclusión que expone el artículo es que la carga viral es determinante en el pronóstico de la infección. ¿Y dónde está la conclusión que ha extraído el autor? Con una nueva pirueta interpreta lo que quiere y como quiere. En el citado artículo se identifican 9 (4%) pacientes de un total de 225 infectados, que han estado asintomáticos durante al menos 7 años (no progresores), con cifras de CD4 estables, y los definen como pacientes "largo tiempo asintomáticos" (LTA). Lógicamente estos pacientes no precisaron recibir tratamiento antirretroviral durante el periodo de estudio ya que se encontraban asintomáticos y estables. De nuevo otra cita que no sostiene las afirmaciones del autor. Ninguno de los estudios que cita ha demostrado que los pacientes no tratados presenten una menor progresión a SIDA que los tratados.

Otro de los trabajos que según el autor demuestra claramente la inutilidad de estos tratamientos (34) está publicado en *The Lancet* (Margaret T May. The Lancet; 2006). En este estudio realizado con 22,217 personas infectadas por el VIH (diagnosticadas entre los años 1995-2003), el autor da a entender que se demuestra que no "hubo reducción en ninguna de las causas de muerte" con los tratamientos antirretrovirales. Para poder establecer esta afirmación habría que comparar la mortalidad en aquellos que no recibieron tratamiento con aquellos que si lo recibieron. Sin embargo, al leer el artículo nos encontramos una nueva sorpresa: todos los pacientes incluidos en el mismo reciben tratamiento

antirretroviral. Lo que intenta demostrar este trabajo no tiene nada que ver con lo que el autor concluye. Dicho estudio muestra es que no se produjo una reducción sustancial de la mortalidad ni en la progresión a SIDA entre aquellos que se diagnosticaron en los años el 1995-96-97 y empezaron a tratarse en esa época y los que se diagnosticaron en los últimos años del estudio (2000-01-02) y empezaron a tratarse entonces. Los autores relacionan este hecho con un menor recuento de CD4 en al diagnóstico en los últimos años, es decir, un diagnóstico más tardío de la infección. Existen cambios epidemiológicos que los autores creen que podrían relacionarse con este hecho (mayor porcentaje de mujeres, mayor incremento del porcentaje de infectados por relaciones heterosexuales frente al de hombres que tienen sexo con hombres, etc.) en los últimos años. Intentar un diagnóstico precoz es uno de los actuales caballos de batalla en el tratamiento de la infección por VIH, pues, como pone de manifiesto el citado trabajo, el retraso diagnóstico es un factor de mal pronóstico. Pero no existe en dicho estudio ningún dato que compare mortalidad entre aquellos que reciben tratamiento y los que no, que permita al autor sostener sus afirmaciones. De nuevo, castillos en el aire.

Existen varios estudios que ponen de manifiesto la reducción de la mortalidad y progresión a SIDA en Europa al comparar la era Pre-TARGA y Post TARGA (35, Fig. 11).

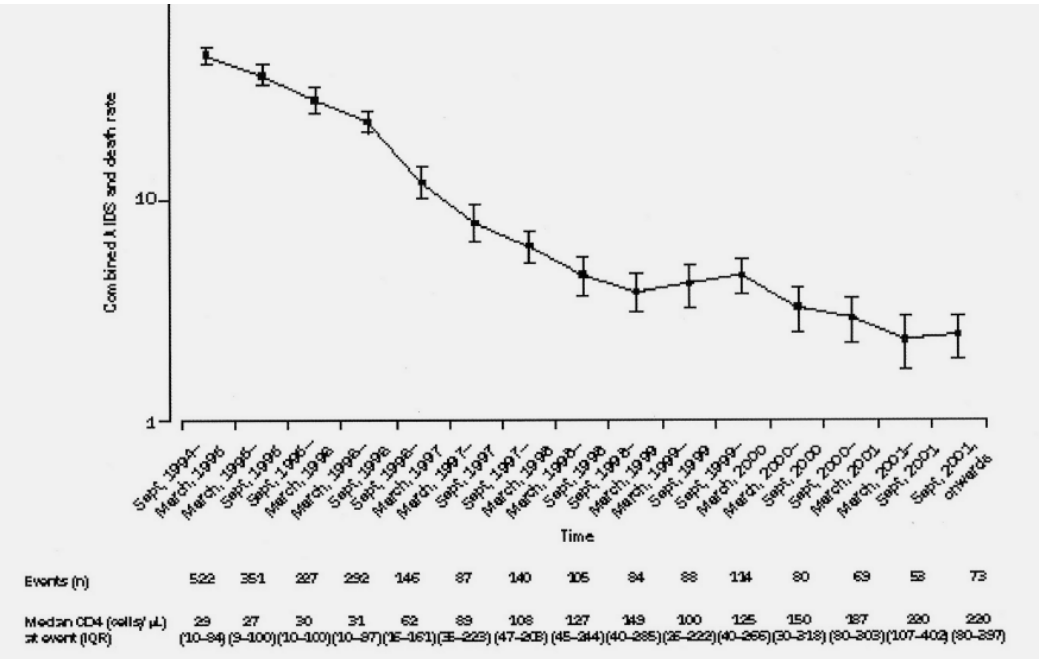


Figura 11. Disminución de mortalidad /Sida en la Cohorte Europea entre 1994-2002(35)

En cuanto al pronóstico de los pacientes infantiles, el cambio que ha supuesto el TARGA ha sido aún mayor que en el caso de los adultos. En el estudio anteriormente comentado realizado en Madrid (23) se ha observado un descenso de la probabilidad de progresión a SIDA o fallecimiento de un 10,57% y un 6,29% respectivamente en la época Pre-TARGA (1990-1996) a un 1, 76% y un 0,36% en la última época (2000-2006) (Figura 6), con un descenso muy marcado de las complicaciones asociadas al VIH (Fig. 12 y 13).

Figura 12. Porcentaje de progresión a muerte y a SIDA entre los niños de la Cohorte de Madrid en los distintos periodos: 1990-96, 1997-99 y 2000-06(30)

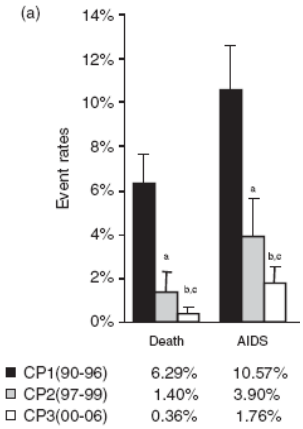


Figura 13: Porcentaje de las distintas complicaciones en pacientes VIH positivos de Niños en la CAM distintos periodos: 1990-96, 1997-99 y 2000-06(30)

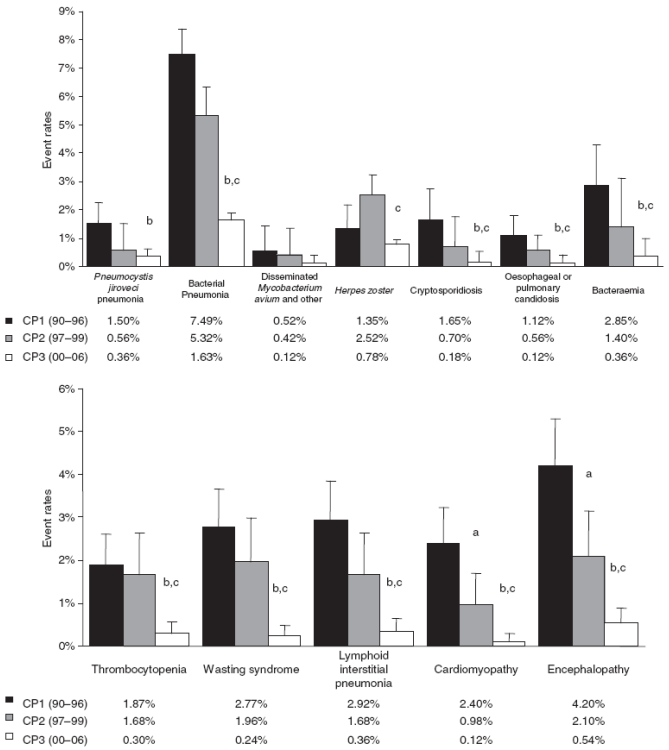


Fig. 3 Opportunistic infections and organ-specific diseases according to calendar period (CP). Statistically significant differences ($P<0.05$) between CPs: a, CP1 and CP2; b, CP1 and CP3; c, CP2 and CP3.

Por último, el estudio CHER (36) que valoraba cuándo se debe empezar la medicación (tratamiento precoz/diferido) en los niños con una infección de transmisión materna, ha demostrado que el inicio precoz reduce la mortalidad del 16% al 4% (ver tabla abajo) y un 76% la progresión a SIDA (Fig. 14 y 15).

Fig. 14

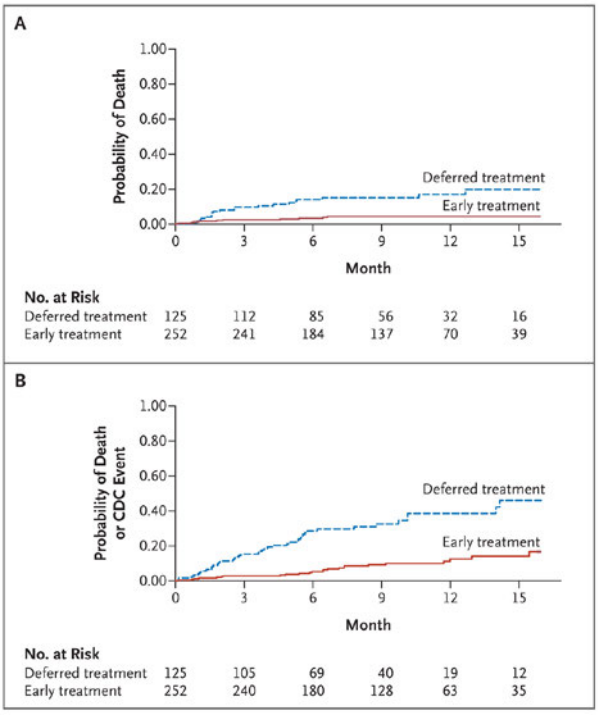


Fig. 15

Adverse Event	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)
Participants with ≥1 event¶	55 (21.8)	55 (44.0)	110 (29.2)
Death	10 (4.0)	20 (16.0)	30 (8.0)
Life-threatening event	9 (3.6)	4 (3.2)	13 (3.4)
New or prolonged hospitalization	41 (16.3)	46 (36.8)	87 (23.1)
Persistent or significant disability or incapacity	0	1 (0.8)	1 (0.3)

*

*** Nota final**: *Con la realización de este dossier pretendíamos alertar al Colectivo Editorial "Virus" y a los posibles lectores de "El Rapto de Higea" de la gran cantidad de errores que aparecen en el capítulo dedicado al VIH. La continua utilización de citas incorrectas que no se corresponden con los argumentos que se exponen en el texto, restan credibilidad al mismo y no deben ser toleradas. Creemos que es necesario realizar un ejercicio de responsabilidad personal y colectiva y confirmar la veracidad de los datos de aquellos materiales que se editan o se distribuyen, especialmente en un tema tan sensible y tan complejo como es la infección por el VIH.*

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HIV treatment response and prognosis in Europe and North America in the first decade of highly active antiretroviral therapy: a collaborative analysis

*The Antiretroviral Therapy (ART) Cohort Collaboration**

Summary

Background Highly active antiretroviral therapy (HAART) for the treatment of HIV infection was introduced a decade ago. We aimed to examine trends in the characteristics of patients starting HAART in Europe and North America, and their treatment response and short-term prognosis.

Methods We analysed data from 22 217 treatment-naive HIV-1-infected adults who had started HAART and were followed up in one of 12 cohort studies. The probability of reaching 500 or less HIV-1 RNA copies per mL by 6 months, and the change in CD4 cell counts, were analysed for patients starting HAART in 1995–96, 1997, 1998, 1999, 2000, 2001, and 2002–03. The primary endpoints were the hazard ratios for AIDS and for death from all causes in the first year of HAART, which were estimated using Cox regression.

Results The proportion of heterosexually infected patients increased from 20% in 1995–96 to 47% in 2002–03, and the proportion of women from 16% to 32%. The median CD4 cell count when starting HAART increased from 170 cells per µL in 1995–96 to 269 cells per µL in 1998 but then decreased to around 200 cells per µL. In 1995–96, 58% achieved HIV-1 RNA of 500 copies per mL or less by 6 months compared with 83% in 2002–03. Compared with 1998, adjusted hazard ratios for AIDS were 1·07 (95% CI 0·84–1·36) in 1995–96 and 1·35 (1·06–1·71) in 2002–03. Corresponding figures for death were 0·87 (0·56–1·36) and 0·96 (0·61–1·51).

Interpretation Virological response after starting HAART improved over calendar years, but such improvement has not translated into a decrease in mortality.

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See Comment page 427

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Introduction

Accurate prognostic information on HIV-1 disease progression after starting highly active antiretroviral therapy (HAART) is important for patients, physicians, and health care providers. In 2002, the Antiretroviral Treatment (ART) Cohort Collaboration published estimates of the probability of disease progression up to 3 years after starting HAART, according to baseline age, transmission risk group, CD4 cell count, viral load, and clinical disease stage before HAART based on over 12 000 patients starting treatment between 1995 and 2000 in Europe, USA, and Canada.[1] Prognosis might improve with time given greater physician experience with HAART, earlier diagnosis, appropriate management of associated toxicities, and the availability of more potent, and less toxic, drugs.[2,3] The increasing availability of combined preparations has reduced the pill burden, which might facilitate patient adherence to regimens.[4,5] Conversely, the emergence of drug-resistant strains of HIV circulating in the infected population and changes in the characteristics of the patients starting HAART could be associated with poorer outcomes.[6,7]

We analysed the updated database of the ART Cohort Collaboration to examine whether patient characteristics at the time of starting HAART, response to therapy, and disease progression have changed over time, using data combined from 12 cohort studies that followed up antiretroviral-naive patients from when they started therapy.

Methods

Patients

The ART Cohort Collaboration is a collaboration of studies from Europe and North America, established with the aim of describing the prognosis of antiretroviral-naive patients starting HAART. The study design has been described in detail elsewhere.[1,8,9] Prospective cohort studies were eligible if they had enrolled at least 100 patients with HIV-1 infection aged 16 years or older who had not previously received antiretroviral treatment; and who had started antiretroviral therapy with a combination of at least three drugs, including nucleoside reverse transcriptase inhibitors, protease inhibitors, or non-nucleoside reverse transcriptase inhibitors (NNRTIs), with a median duration of follow-up of at least 1 year. All cohorts provided data, which had been made anonymous, for a predefined set of demographic, laboratory, and clinical variables.

The database was updated in 2004 to include patients who had started HAART between 2000 and 2003. 12 cohorts contributed data: the French Hospital Database on HIV (FHDH) ANRS CO4[10] and the Aquitaine Cohort[11] ANRS CO3 (France), the AIDS Therapy Evaluation project Netherlands (ATHENA),[12] Italian Cohort of Antiretroviral-Naive Patients (ICONA),[13] Swiss HIV Cohort Study (SHCS),[14] Frankfurt HIV Cohort[15] and Köln/Bonn Cohort[16] (Germany), the EuroSIDA study (20 countries in Europe and Argentina),[17] the Collaborations in HIV Outcomes Research US (CHORUS, USA),[18] the Royal Free

Hospital Cohort (UK),[19] the British Columbia Centre for Excellence in HIV/AIDS,[20] and the South Alberta Clinic (Canada).[21]

Statistical analysis

Analyses were stratified by calendar year of starting HAART, with the earliest and latest years (1995–96 and 2002–03) grouped because fewer patients started treatment in these periods. Response to therapy 6 months after starting HAART was measured by the proportion of patients reaching an HIV-1 RNA viral load of 500 copies per mL or less, and by change in CD4 cell count from baseline in patients with available measurements. As pre-specified in the data collection protocol, the measurements of CD4 cell count and HIV-1 viral load nearest to 6 months and between 3 and 9 months after the start of treatment (median time of measurement 5·8 months, IQR 5·2–6·6 months) were used. Multivariable logistic regression models were used to estimate the odds ratios of undetectable viral load at 6 months after starting therapy for each calendar year. In all analyses, the comparator year was 1998; before that time HAART was rapidly evolving, whereas from 1998 onwards both protease inhibitor-based and NNRTI-based HAART were available.

We examined clinical prognosis based on two endpoints: firstly AIDS events (including AIDS-related deaths), and secondly death from all causes. Kaplan-Meier estimates of the probability of these two endpoints up to 1 year after starting HAART were graphed by calendar year of starting. Cox proportional hazard models were used to estimate the crude and adjusted hazard ratio of these two endpoints for each calendar year. Models were adjusted for age, sex, transmission risk group, baseline CD4 cell count and viral load, and pre-HAART Centers for Disease Control and Prevention (CDC) disease stage, and were stratified by cohort. All patients were censored at 1 year after starting HAART in these analyses. In sensitivity analyses, we censored follow-up at 2 years after start of HAART, and estimated hazard ratios for the combined outcome of AIDS or death from all causes, because misclassification of deaths could lead to underestimation of the number of AIDS events. We also examined AIDS outcomes grouped as tuberculosis and non-tuberculosis AIDS.

We used Stata software version 9.0 for analyses. Results are presented as Kaplan-Meier estimates of the probability of patients reaching an endpoint, and odds ratios or hazard ratios with 95% CIs.

Role of the funding source

No funding source had any involvement in the study design, in the collection, analysis, and interpretation of data, writing of the report, or the decision to submit the paper for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Data for 22 217 patients who were aged 16 years and over, were antiretroviral naive before starting HAART, and who started therapy between 1995 and 2003, were available for analyses. 19 560 (88%) patients had CD4 cell counts and 19 164 (86%) viral load measurements at 6 months. Table 1 shows patient characteristics at baseline by calendar year of starting HAART. The median age at

	Patients (n)	Age (years)	Women	CD4 cell count (cells per μL)	Log_{10} viral load	AIDS diagnosis	Transmission risk group			Initial HAART regimen		
							Men who have sex with men	Heterosexual	Injecting drug user	Protease inhibitor based	NNRTI-based	Four or more drugs*
1995–96	1232	36 (32–43)	197 (16%)	170 (67–320)	5·0 (4·4–5·5)	386 (31%)	684 (56%)	250 (20%)	166 (13%)	1169 (95%)	19 (2%)	12 (1%)
1997	4785	35 (31–42)	968 (20%)	267 (118–411)	4·9 (4·3–5·4)	959 (20%)	2148 (45%)	1323 (28%)	951 (20%)	4461 (93%)	258 (5%)	121 (3%)
1998	4583	36 (31–42)	1056 (23%)	269 (110–428)	4·8 (4·1–5·3)	970 (21%)	1802 (39%)	1509 (33%)	896 (20%)	3780 (82%)	769 (17%)	142 (3%)
1999	3699	36 (31–43)	888 (24%)	250 (102–405)	4·8 (4·2–5·3)	825 (22%)	1418 (38%)	1362 (37%)	596 (16%)	2054 (56%)	1464 (40%)	234 (6%)
2000	3203	37 (31–43)	919 (29%)	209 (86–353)	4·9 (4·3–5·4)	769 (24%)	1132 (35%)	1322 (41%)	448 (14%)	1430 (45%)	1479 (46%)	237 (7%)
2001	2783	37 (31–43)	845 (30%)	198 (86–316)	5·0 (4·3–5·4)	689 (25%)	938 (34%)	1227 (44%)	329 (12%)	1237 (44%)	1126 (40%)	186 (7%)
2002–03	1932	37 (31–43)	613 (32%)	202 (90–310)	4·9 (4·4–5·4)	477 (25%)	655 (34%)	917 (47%)	167 (9%)	864 (45%)	767 (40%)	218 (11%)
Total	22 217	36 (31–43)	5486 (25%)	234 (98–380)	4·9 (4·3–5·4)	5075 (23%)	8777 (40%)	7910 (36%)	3553 (16%)	14995 (67%)	5882 (26%)	1150 (5%)

IQR=interquartile range. *Counting ritonavir-boosted protease inhibitors as one drug. Data are median (IQR) or n (%), unless otherwise specified.

Table 1: **Patient characteristics at baseline by calendar year of starting HAART, ART-CC, 2004**

starting HAART changed little over calendar time, but the proportion of female patients increased from 16% in 1995–96 to 32% in 2002–03. There were substantial changes in the proportions of patients in the major presumed transmission groups. 56% of patients starting HAART in 1995–96 were presumed to have been infected via male homosexual contact: this percentage decreased to 34% by 2002–03. By contrast, the proportion of patients infected via heterosexual contact increased from 20% in 1995–96 to 47% in 2002–03. The percentage of patients infected via injection drug use declined from 20% in 1997 to 9% in 2002–03. The remaining patients were infected through contact with contaminated blood (less than 1%) or the mode of transmission was not specified (around 9%).

The median CD4 cell count when starting HAART increased from 170 cells per μL in 1995–96 to 269 cells per μL in 1998 but then decreased to around 200 cells per μL. During 1995–98 most patients started a protease inhibitor-based HAART regimen whereas, from 1999 onwards, at least 40% started HAART with NNRTI-based regimens. The proportion of patients starting HAART with four or more drugs (counting ritonavir-boosted protease inhibitors as one drug) increased from 1% in 1995–96 to 11% in 2002–03.

Table 2 shows virological and immunological response to HAART by calendar year of starting HAART. In 1995–96, 58% of patients achieved an HIV-1 RNA of 500 copies per mL or less by 6 months; this increased to 73% in 1997 and 83% in 2002–03. Median post-HAART change in CD4 cell count at 6 months was slightly lower in 1995–96 compared with later years. Table 3 shows adjusted odds ratios for reaching HIV-1 RNA of 500 copies per mL or less at 6 months after starting HAART, by calendar year of starting HAART for all patients and separately for the three major transmission risk groups. Compared with 1998 (the reference year), the odds ratio was 0·38 (95% CI 0·33–0·44) in 1995–96 and rose to 1·51 (1·28–1·77) in 2002–03. The change in the odds of HIV-1 RNA being 500 copies per mL or less with calendar time was greater for men who have sex with men, with an odds ratio of 0·31 (0·25–0·38) in 1995–96 increasing to 2·11 (1·51–2·94) in 2002–03. By contrast, for injecting drug users the odds ratio was 0·61 (0·41–0·91) in 1995–96, increasing to 1·67 (1·23–2·27) in 2000, and then decreasing to 1·09 (0·69–1·72) in 2002–03. Odds ratios for patients infected heterosexually were much the same as average values. We used a likelihood ratio test comparing regression models with and without interaction terms to test formally for interaction between transmission risk group and linear trend over time in the odds of achieving HIV-1 RNA of 500 copies per mL or less. The results suggested that different groups attained varying improvements in viral suppression rates over time (p=0·02). Women had similar results to men once transmission risk group was accounted for.

The figure shows Kaplan-Meier estimates of the cumulative proportion of AIDS (top) and death (bottom) for the first year after starting HAART, separately for time periods 1995–97, 1998–99, and 2000–03. The figure shows a lower proportion in 1998–99 than in either the earlier or later years. The estimated probability of death up to 1 year after starting HAART did not differ greatly by calendar period. Table 4 shows crude and adjusted hazard ratios from multivariable Cox models, for AIDS and for death from all causes, by calendar year. Compared with 1998, the adjusted hazard ratio for AIDS was 1·30 (95% CI 1·09–1·54) in 1997 and 1·35 (1·06–1·71) in 2002–03. There was some evidence that AIDS trends over time differed between transmission risk groups; compared with 1998, the adjusted hazard ratios in 2002–03 were 1·18 (0·78–1·78) for men who have sex with men, 1·52 (1·07–2·16) for heterosexually infected patients, and 1·73 (0·84–3·55) for injecting drug users (webtable 1). However, CIs were wide and the test for interaction between transmission risk group and linear trend over time gave p=0·24. Adjusted mortality hazard ratios did not differ greatly with calendar year (table 4).

See Online for webtable 1

	Patients with viral load measurement	Patients with viral load ≤500 copies per mL	Patients with CD4 measurement	CD4 cell count (cells per μL)	Increase in CD4 cell count (cells per μL)
1995–96	1046 (85%)	607 (58%)	1101 (89%)	275 (153–436)	90 (23–173)
1997	4140 (87%)	3029 (73%)	4244 (89%)	383 (220–565)	108 (30–202)
1998	4032 (88%)	3061 (76%)	4099 (89%)	382 (214–582)	106 (30–198)
1999	3213 (87%)	2608 (81%)	3261 (88%)	364 (210–543)	102 (30–196)
2000	2794 (87%)	2282 (82%)	2856 (89%)	326 (184–498)	100 (36–189)
2001	2478 (89%)	2020 (82%)	2517 (90%)	312 (190–467)	105 (40–183)
2002–03	1461 (76%)	1218 (83%)	1482 (77%)	310 (184–468)	104 (40–184)
Total	19164 (86%)	14825 (77%)	19560 (88%)	349 (200–528)	103 (32–192)

The percentage of patients with viral load 500 copies per mL or less is taken from those with a measurement at 6 months. Data are n (%) or median (IQR).

Table 2: **Treatment response at 6 months after initiating therapy by calendar year of starting HAART, ART-CC, 2004**

	All	Transmission risk group		
		Men who have sex with men	Heterosexual	Injection drug use
1995–96	0·38 (0·33–0·44)	0·31 (0·25–0·38)	0·41 (0·30–0·57)	0·61 (0·41–0·91)
1997	0·82 (0·74–0·90)	0·72 (0·61–0·85)	0·88 (0·74–1·06)	0·86 (0·69–1·07)
1998 (reference year)	1	1	1	1
1999	1·28 (1·14–1·44)	1·18 (0·97–1·44)	1·39 (1·15–1·69)	1·07 (0·83–1·39)
2000	1·40 (1·24–1·58)	1·40 (1·13–1·74)	1·28 (1·06–1·55)	1·67 (1·23–2·27)
2001	1·33 (1·17–1·52)	1·40 (1·10–1·76)	1·29 (1·05–1·57)	1·29 (0·92–1·80)
2002–03	1·51 (1·28–1·77)	2·11 (1·51–2·94)	1·33 (1·06–1·67)	1·09 (0·69–1·72)

Results from logistic regression models. All analyses were adjusted for age, sex, baseline CD4 cell count and viral load, stage, and cohort.

Table 3: **Odds ratios (95% CI) for reaching HIV-1 RNA concentrations ≤ 500 copies per mL at 6 months after starting HAART, by calendar year of starting HAART, ART-CC, 2004**

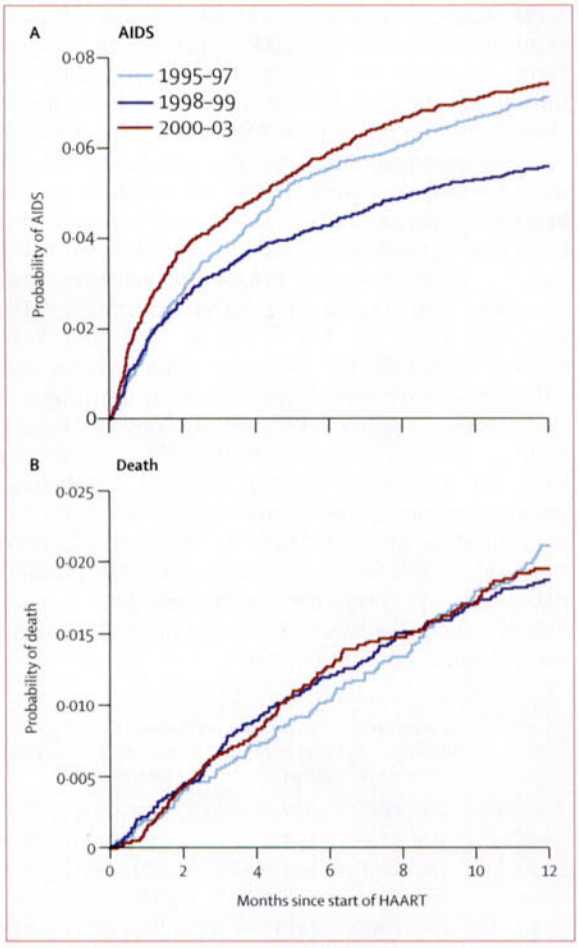

Figure: **Kaplan-Meier estimates of cumulative proportion of (A) AIDS and (B) death by calendar year of starting HAART, ART-CC, 2004**

In sensitivity analyses, the trend over calendar time in hazard ratios for the combined endpoint of AIDS or death was less marked than the trend for AIDS alone. For example, the adjusted hazard ratios comparing 2002–03 with 1998 were 1·26 (1·01–1·58) for AIDS or death but 1·35 (1·06–1·71) for AIDS (webtable 2). The estimated hazard ratios from models in which follow-up was censored at 2 years after start of treatment were much the same as the reported estimates based on 1 year of follow-up (webtable 3).

See Online for webtables 2 and 3

We investigated whether the increase in AIDS events in the most recent years was attributable to an increase in tuberculosis incidence. In the analysis with tuberculosis as outcome, follow-up time was censored at non-tuberculosis AIDS events, and vice versa. Table 5 shows the crude and adjusted hazard ratios separately for tuberculosis and non-tuberculosis AIDS for all patients. The analysis shows that the increase in AIDS in 2002–03 compared with 1998 is largely attributable to an increase in tuberculosis; the adjusted hazard ratio for tuberculosis was 2·94 (1·70–5·08) compared with 1·15 (0·88–1·50) for non-tuberculosis AIDS.

Discussion

The results of this collaborative study, which involved 12 prospective cohorts and over 20 000 patients with HIV-1 from Europe and North America, show that the virological response after starting HAART has improved steadily since 1996. However, there was no corresponding decrease in the rates of AIDS, or death, up to 1 year of follow-up. Conversely, there was some evidence for an increase in the rate of AIDS in the most recent period. These trends were accompanied by changes in the characteristics of patients starting HAART. In the early years when HAART was being introduced, most patients were men who have sex with men, but by 2002–03 most patients starting HAART had been infected through heterosexual transmission. Over the same time, the proportion of female patients doubled. The median CD4 cell count when starting HAART has declined in recent years.

The discrepancy between the clear improvement we recorded for virological response and the apparently worsening rates of clinical progression might be related to the change in the demographic characteristics of study participants, with an increasing number of patients from areas with a high incidence of tuberculosis. For example, in the Swiss HIV Cohort Study[14] there was a steady increase in the number of patients from sub-Saharan Africa.[22] These patients were younger, more likely to be female, and more likely to have been infected heterosexually than other study participants. Also, they had lower CD4 cell counts at

	AIDS				Death			
	Patients n	Events n (%)	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)	Patients n	Deaths n (%)	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
1995–96	1096	103 (9%)	1·55 (1·22–1·97)	1·07 (0·84–1·36)	1232	27 (2·2%)	1·20 (0·77–1·87)	0·87 (0·56–1·36)
1997	4460	287 (6%)	1·23 (1·03–1·46)	1·30 (1·09–1·54)	4785	98 (2·1%)	1·13 (0·85–1·52)	1·12 (0·84–1·51)
1998 (reference)	4222	222 (5%)	1	1	4583	85 (1·9%)	1	1
1999	3328	192 (6%)	1·08 (0·89–1·32)	1·07 (0·88–1·30)	3699	67 (1·8%)	1·00 (0·72–1·38)	0·93 (0·67–1·29)
2000	2873	204 (7%)	1·35 (1·11–1·63)	1·18 (0·97–1·43)	3203	63 (2·0%)	1·06 (0·76–1·47)	0·93 (0·67–1·29)
2001	2421	172 (7%)	1·35 (1·10–1·65)	1·23 (1·00–1·50)	2783	49 (1·8%)	1·02 (0·71–1·45)	0·87 (0·61–1·24)
2002–03	1656	105 (6%)	1·46 (1·15–1·85)	1·35 (1·06–1·71)	1932	25 (1·3%)	1·09 (0·69–1·71)	0·96 (0·61–1·51)

Table 4: **Crude and adjusted hazard ratios for AIDS and death by year of starting HAART, ART-CC, 2004**

presentation, and the most frequent AIDS-defining event was tuberculosis.[22] Similar trends have been seen in other European countries and in North America.[23–25] In the USA, the rates of tuberculosis are increasing in foreign-born people, and outbreaks are increasingly common in other groups at high risk of HIV infection, including prisoners,[26] homeless people,[27] and gay, transvestite, and transsexual HIV-infected men.[28] Immune reconstitution disease, an adverse consequence of restoration of pathogen-specific immune responses, might also be a problem, particularly in those infected with tuberculosis. This disease could have become more common in later years with the occurrence of more rapid reduction in viral replication and increase in CD4 cells due to the use of more potent antiretroviral drugs.[29] The increasing number of heterosexually infected immigrants and refugees cannot fully explain the trends seen in our study. The same trends in the rate of AIDS were also present, although somewhat weaker, in men who have sex with men. Also, although the average CD4 cell count at baseline varied by transmission risk group, the same pattern of increase and decline with calendar periods was seen for each risk group, and for both sexes. We noted that the median time to the first AIDS event after starting HAART decreased over time.

In this collaborative study, AIDS diagnoses are not centrally reviewed or verified. The increase might thus be artifactual if ascertainment has become more complete in recent years. This might have been particularly true for tuberculosis, because of the growing awareness of physicians about co-infection.[25,30,31] However, ascertainment bias is unlikely, because all cohorts use the same criteria for the prospective diagnosis of AIDS-defining events,[32] and study clinics are based in specialised centres with extensive expertise in HIV medicine. Worse outcomes are also unlikely to be due to more drug-resistant strains of HIV in the population, as viral suppression at 6 months improved over calendar time, and the analysis was restricted to the first year of HAART. Indeed, improvements in viral suppression could conceivably translate into reduced rates of AIDS and death later on.

Unlike previous studies that have looked at changes in survival by calendar period,[33,34] all patients in this study were followed-up from initiation of HAART and had not been previously exposed to antiretroviral therapy. Results are therefore not confounded by previous antiretroviral treatment. The database included patients from many countries in Europe and North America who started HAART in different settings since 1995. The spectrum of patients was broad: men and women, teenagers and elderly people were included, and the major exposure categories were well represented. The severity of immunodeficiency at baseline ranged from severe to non-existent, and viral replication from undetectable to extremely high. Our results should therefore be generalisable to other settings.

Limitations include the lack of data for ethnicity or country of origin. Information on immigrants is not obtained routinely in the studies participating in the ART Cohort Collaboration, and their contribution in the context of the trends seen could not be examined directly. However, we note that tuberculosis largely accounted for the reported increase in AIDS events. Also, we do not have adequate information on causes of death for all patients, which means that we are unable to discern whether the stability of mortality rates over time results from reductions in AIDS-related mortality being offset by an increase in competing non-HIV related causes of death.[35] Finally, our results might be affected by selection bias because 12% and 14% of patients had missing CD4 cell counts and viral load measurements, respectively, at 6 months. The results are consistent with those from another multi-cohort analysis,[36] which included studies not represented in the present collaboration.

The improvement in virological response was most pronounced in men who have sex with men, and less noticeable in heterosexually infected patients. In patients with a history of injecting drug use the picture was more complex, with an initial improvement followed by a worsening of virological response in later years. In earlier years, patients infected via injecting drug use might have been selected on the basis of their likely adherence to therapy, whereas such selection might have been less pronounced in more recent years. Clearly, the reasons why injecting drug users and heterosexually infected patients do not seem to achieve the same treatment response as do men who have sex with men need to be examined and strategies to improve outcomes developed and implemented.

The decline of CD4 cell count when starting HAART in recent years must also be of concern. Patients starting

	Patients n	Events n (%)	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
Tuberculosis				
1995–96	1096	8 (0·7%)	1·07 (0·49–2·34)	0·73 (0·33–1·63)
1997	4460	27 (0·6%)	0·88 (0·52–1·49)	0·97 (0·57–1·63)
1998	4222	29 (0·7%)	1	1
1999	3328	34 (1·0%)	1·50 (0·91–2·46)	1·50 (0·91–2·48)
2000	2873	38 (1·3%)	1·95 (1·20–3·16)	1·69 (1·04–2·75)
2001	2421	28 (1·2%)	1·75 (1·04–2·94)	1·48 (0·87–2·50)
2002–03	1656	26 (1·6%)	3·20 (1·88–5·45)	2·94 (1·70–5·08)
Other AIDS defining conditions				
1995–96	1096	94 (9%)	1·87 (1·46–2·39)	1·09 (0·84–1·40)
1997	4460	261 (6%)	1·27 (1·05–1·53)	1·34 (1·11–1·61)
1998	4222	195 (5%)	1	1
1999	3328	160 (5%)	1·05 (0·85–1·29)	1·01 (0·82–1·25)
2000	2873	170 (6%)	1·29 (1·05–1·59)	1·12 (0·91–1·37)
2001	2421	146 (6%)	1·34 (1·08–1·67)	1·19 (0·96–1·48)
2002–03	1656	81 (5%)	1·35 (1·04–1·75)	1·15 (0·88–1·50)

Results from Cox regression models (crude and adjusted for age, sex, CD4 cell count, viral load, stage, risk group), stratified by cohort. Follow-up was censored at 1 year after starting HAART.

Table 5: **Crude and adjusted hazard ratios for tuberculosis and AIDS defining conditions other than tuberculosis, ART-CC, 2004**

treatment with CD4 count less than 200 cells per µL are at higher risk of disease progression and death in the long term compared with those with higher baseline CD4 cell counts.[1] Early diagnosis and treatment is therefore of great importance to prevent clinical progression. A survey of new HIV diagnoses in the UK and Ireland showed that many opportunities for earlier diagnosis are missed.[37] Our results indicate that such oversights could be common in many countries and settings, and that therefore an expansion of voluntary and cost-effective screening in health-care settings is likely to be beneficial.[38] The ART Cohort Collaboration will continue to monitor the characteristics and prognosis of HIV-infected patients starting HAART and update analyses at regular intervals.

Contributors

M Egger conceived the ART Cohort Collaboration and wrote the original proposal with B Ledergerber, J Lundgren, J Sterne, and A Phillips. All authors contributed to the final version of the protocol. M May and J Sterne did statistical analyses. M May, J Sterne, D Costagliola and M Egger wrote the first draft of the paper. C Sabin, A Phillips, A Justice, F Dabis, J Gill, J Lundgren, R Hogg, F de Wolf, G Fätkenheuer, S Staszewski, and A d'Arminio Monforte contributed to discussions on statistical analyses and to writing the paper.

Conflict of interest statement

We declare that we have no conflict of interest.

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Predictors for Non- and Slow Progression in Human Immunodeficiency Virus (HIV) Type 1 Infection: Low Viral RNA Copy Numbers in Serum and Maintenance of High HIV-1 p24-Specific but Not V3-Specific Antibody Levels

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To gain insight into determinants that define the duration of the asymptomatic period preceding AIDS, groups of long-term asymptomatic (LTA) person (>7 years of follow-up) and slow and rapid progressors of human immunodeficiency virus infection were studied. LTAs had no clinical manifestations of AIDS or immunologic abnormalities in 7 years of follow-up. RNA copy numbers, *gag*- and *env*-specific, and neutralizing antibody titers in serum were determined 1 and 5 years after seroconversion or entry into the cohort. Early in infection, before immunologic markers or clinical manifestations allowed group discrimination, subjects who were later classified as LTAs had significantly less serum viral RNA than progressors. No significant increase in virus load was found in progressors, indicating that the initial load defines clinical outcome. In slow progressors, high virus load was associated with high p24-specific antibody titers, suggesting that delay of clinical manifestations of AIDS may be related to the presence of high levels of p24-specific but not V3-specific antibodies.

Since the beginning of the AIDS pandemic, the mechanisms involved in disease progression have been a major focus of research. Results of longitudinal cohort studies have shown that progression is associated with several immunologic and virologic changes, such as a decline in $CD4^+$ cell count, serum p24 antigenemia, emergence of more cytopathic virus variants (switch from nonsyncytium inducing [NSI] to syncytium inducing [SI]), and increased levels of $ß_2$-microglobulin and virus burden, that are now used as predictors for disease progression [1–16]. Disease progression has been thoroughly investigated, but there may still be valuable information to learn from people who remain symptomless after infection. Many cohort studies were started during the early and mid-1980s. Long-term asymptomatic (LTA) participants of those studies could be identified only after 8–10 years.

It is not known whether a relatively benign course of infection is due to viral or host factors or both. Recently, some studies reporting data on human immunodeficiency virus (HIV) type 1–infected persons remaining symptom-free for prolonged periods have been published. Lifson et al. [17] compared a group of 24 seropositive nonprogressors with a control group who had opportunistic infections or Kaposi's sarcoma. Learmont et al. [18] did a follow-up study of 6 subjects who acquired HIV-1 infection from transfusions of blood from the same donor. The persistent disease-free status of the nonprogressors was suggested to be due to stimulation of the immune system [17] or to low virulence of the transmitted HIV-1 strain [18]. Sheppard et al. [19] studied several laboratory markers but could not distinguish the nonprogressors as a distinct subgroup from other seropositive persons; therefore, they suggested that nonprogressors are undergoing slow HIV-1 disease progression.

We studied the role of virologic and immunologic factors in a group of LTAs participating in the Amsterdam cohort study [3, 20, 21] or visiting the Academic Medical Center (AMC) AIDS clinic (University of Amsterdam). These LTAs did not progress to clinical manifestations of AIDS and showed no immunologic abnormalities, as measured by $CD4^+$ cell counts and T cell activation by CD3/TCR antibodies, within 7 years of follow-up. LTAs with persistently normal $CD4^+$ cell counts (>400 $CD4^+$ cells/μL), normal T cell activity (anti-CD3 response >1000 cpm), absence of p24 antigen, and presence of p24 antibodies and NSI virus variants were compared with persons who progressed to AIDS (progressors). Two groups of progressors were distinguished: slow progressors were clinically and immunologically comparable to LTAs for 4 years but, thereafter, showed decreasing amounts of $CD4^+$ cells, declining T cell activity,

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Table 1. $CD4^+$ cell counts, T cell activity, and virus phenotype in long-term asymptomatic (LTA) human immunodeficiency virus–infected persons and those progressing to AIDS.

	LTAs (n = 11)	Progressors		Seronegative controls (n = variable)*
		Slow (n = 6)	Rapid (n = 6)	
Follow-up†	8.7 ± 0.6	6.1 ± 1.0	3.2 ± 1.3	—
CD4‡				
Year 1	786 ± 160	803 ± 199	782 ± 320	710 ± 270
Year 4	792 ± 259	427 ± 133	380 ± 209	740 ± 220
Year 5	737 ± 293	322 ± 152	372 ± 237	770 ± 380
Decline/year, years 1–5	−25 ± 79	−120 ± 67	−118 ± 83	—
Anti-CD3, mean cpm (range)				
Year 4	4035, (1370–14330)	1600, (740–3670)	460, (30–1300)	4208, (460–14080)
Year 5	5530, (2610–11480)	353, (90–2270)	315, (200–1240)	5542, (1450–10630)
Phenotype				
NSI	11	4	6	—
SI	0	2	0	—

NOTE. NSI, nonsyncytium inducing; SI, syncytium inducing.

* For $CD4^+$ cell count, n = 48, 28, and 27, respectively, for years 1, 4, and 5; for anti-CD3 response, n = 26 and 23, respectively, for years 4 and 5.

† Mean years follow-up to study end (October 1993) or to first diagnosis of AIDS.

‡ Mean ± SD $CD4^+$ cells/μL blood.

and clinical symptoms of AIDS; rapid progressors progressed to AIDS in <4 years. To determine the mechanisms involved in long-term survival of HIV-1–infected persons, we studied sera from LTAs and progressors. The sera, which were obtained early (1 year) and late (5 years) after seroconversion or entry into the cohort study, were tested for viral RNA load, p24 antibody titers, reactivity to peptides corresponding to the V3 domain of naturally occurring HIV-1 strains and to gp41, and for neutralization of HIV-1 laboratory strains (MN, HXB3, and RF).

Patients and Methods

Subjects. Study members were homosexual men participating in the Amsterdam cohort study on the natural course of HIV-1 infection [3, 20, 21] or visiting the AMC AIDS clinic. In December 1992, we selected 3 groups of persons, all positive for p24 antibodies and negative for p24 antigen and either not or slowly or rapidly progressing to AIDS. To be considered LTA, subjects had to be asymptomatic for ≥7 years (Centers for Disease Control and Prevention class II [asymptomatic] or III [lymphadenopathy] [22]) and have a $CD4^+$ cell count persistently >400 cells/μL and T cell activity >1000 cpm. Of 225 seropositive subjects, 9 (4%) met these criteria and were considered LTAs (table 1). In addition, 2 persons visiting the AMC AIDS clinic and complying with the criteria were added to the LTA group.

Two groups of controls were selected from the Amsterdam cohort: A group of slow progressors was selected for being clinically and immunologically comparable to the LTAs for 4 years ($CD4^+$ cell counts >400/μL and anti-CD3 response >1000 cpm), followed by a decline of their $CD4^+$ cell counts or T cell activity and development of AIDS within 7 years; 6 subjects met these criteria. A group of rapid progressors was selected with known seroconversion dates, having a decline of $CD4^+$ cell counts or T cell activity (or both), and progressing to AIDS within 4 years after seroconversion. Since all LTAs had NSI virus variants during the study, the rapid progressors were selected as subjects with NSI variants in the period before diagnosis of AIDS (table 1); 6 subjects met these criteria.

All but 3 participants were of Dutch origin, the others (all LTAs) came from Spain (H8), Uruguay (H337), or Venezuela (RV). There was no significant difference among groups in mean age (41.4 ± 4.6, 42.8 ± 5.7, and 40.0 ± 8.7 years for LTAs and slow and rapid progressors, respectively).

Serologic data. Sera were screened for the presence of antibodies to HIV-1 with commercial ELISA (Abbott Laboratories, Abbott Park, IL) and confirmed by Western blot (Diagnostic Biotechnology, Herent, Belgium). End-point titers of antibodies to HIV-1 core proteins were measured using a newly developed EIA for the detection and semiquantitation of antibodies to p24 core protein (Abbott). Polystyrene beads coated with recombinant p24 antigen were incubated with patient sera. Antibodies to p24 were quantitated in terms of end-point titer, which was defined as the reciprocal serum dilution at which the optical density was equal to the cut-off value of 84 sera obtained from seronegative persons plus four times the SD.

HIV-1 p24 antigen was detected using a solid-phase, sandwich-type immunoassay (Abbott).

$CD4^+$ cell counts and T cell activity. The 3 groups were distinguished on the basis of distinctions in $CD4^+$ cell counts and T

cell activity. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using density-gradient centrifugation on ficoll-hypaque. $CD4^+$ cells were enumerated by indirect immunofluorescence using monoclonal antibodies and flow cytometry (Epics C; Coulter Electronics, Luton, UK).

As a marker for T cell responsiveness, anti-CD3 reactivity was measured in triplicate as described by Schellekens et al. [23]. The anti-CD3 response is given as counts per minute (cpm). Previous studies showed that cpm and reactivity to anti-CD3 per lymphocyte are linearly correlated [24].

Detection of virus phenotype. To determine the biologic phenotype of the virus variants present in the peripheral blood, patient PBMC were cocultivated with the $CD4^+$ T cell line MT-2 as described by Koot et al. [25].

Nucleic acid isolation and quantitative nucleic acid sequence–based amplification. Nucleic acids were isolated from 100 μL of serum as described [26, 27]. HIV-1 RNA in serum was quantified by competitive coamplification of a dilution series of in vitro–generated RNA using nucleic acid sequence–based amplification technology [26, 28, 29]. The in vitro RNA (Q-RNA), comprising the *gag* and part of the *pol* region, differs only by a sequence randomization of 20 nucleotides from the wild-type RNA, thus ensuring equal efficiency of amplification. Tenfold dilutions ranging from 10^2 to 10^6 molecules of Q-RNA were mixed with 2 μL of aliquoted nucleic acid samples isolated from patient sera and subjected to amplification. Wild-type and mutant (Q) amplification ratios were determined in a bead-based colorimetric assay [30].

Specificity of human antibody response to V3 and gp41 domains. The method for determining the specificity of human antibodies to the V3 and gp41 regions of HIV-1 has been described by Zwart et al. [31]. A peptide panel composed of peptides that were 16–17 residues in length was used, covering the neutralization V3 domain of gp120 of European and American [31] and of African virus strains [32] (table 2). As a control, we used a peptide covering a constant epitope of gp41 (table 2), previously described by Norrby et al. [33]. All peptides were produced using Merrifield solid-phase synthesis (European Veterinary Laboratory, Woerden, Netherlands). For end-point titration of the sera, 2-fold dilutions, starting from 1:100, were tested using microtiter plates coated with 100 ng/well. All assays were done in duplicate on two separate microtiter plates. If reactivity of a serum to a peptide was >10% higher than reactivity of this serum to any of the other peptides from the panel, the serum was considered to show specificity for that peptide.

Virus stock preparation and neutralization assays. Virus stocks of HIV-1 MN, HXB3, and RF were prepared by cell-free infection of H9 cells. Infectious titers were determined by end-point titration on the basis of syncytium formation using the C8166 cell line as described [34, 35].

Cell-free neutralization was done as described [34, 35]. In brief, a virus-stock dilution containing 100 $TCID_{50}$ was incubated with serial dilutions of heat-inactivated human serum. C8166 cells were added and incubated for 5–7 days and scored for the presence of syncytia. The neutralizing activity against the HIV-1 strains was expressed as the reciprocal of the highest antibody dilution giving 99% inhibition of syncytium formation.

Statistical analyses. The Mann-Whitney test was used to analyze data concerning RNA copy numbers, p24-, V3-, and gp41-specific antibody titers, and neutralization titers obtained from the 3 different groups. For comparison of the data of each group obtained early and late in infection, paired Student's *t* tests were used after log transformation. For RNA copy numbers below the level of detection, the amount of viral RNA was fixed at 10^4 RNA copies/mL.

Fisher's exact test was used to calculate the correlation between virus load and p24 antibody titers. To determine the relative risk of developing AIDS in the presence of detectable virus load and low antibody titer, all subjects with detectable virus load at 1 year were grouped on the basis of their p24 antibody end-point titers. Subjects with low antibody titers had end-point titers below the median (median end-point titer, 1466); subjects with high antibody titers were those with titers above the median.

Table 2. Alignment of the V3 synthetic peptides to the MN-like and African-like sequences and the sequence of the gp41 peptide.

Peptide	V3 sequence	Region
	304 319	
p108	RKSIHIGPGRAFYTTG	Eur/US
p109	----N----------	Eur/US
p110	----P----------	Eur/US
p111	-RR-TM----VL----	Eur/US
p112	SRG-R------ILA-E	Eur/US
p113	-R--YT------H--D	Eur/US
p114	----S-------F---	Eur/US
p115	--R-TM----VY----	Eur/US
p117	--G-F-----NI----	Eur/US
p116	-R---V---Q---A--	Africa
p164	-QGT-------Y---R	Africa
p168	---V-----Q---A--	Africa
p169	-E-VR----QT--A--	Africa
p170	-Q-TR----Q-L--NK	Africa
p172	---V-----QTS-A--	Africa
	gp41 sequence	
	589 606	
p149	DQQLLGIWGCSGKLICTT	

NOTE. EUR = Europe; US = United States.

Results

Characteristics of study population. At the start of this study, 680 men (455 seronegative, 225 seropositive) of our cohort were in active follow-up. Nine (4%) of the seropositive subjects met the stringent LTA criteria. Two patients visiting the AMC AIDS clinic who also met the criteria were added to the group. The mean follow-up, until the end of the study in October 1993, was 8.7 ± 0.6 years (table 1). One subject (H709) had a known date of seroconversion; the other sub-

jects were seropositive at entry into the cohort. The 2 AMC AIDS clinic patients were seropositive at their first visit to the clinic. Phenotyping of the viruses present in the peripheral blood revealed that all LTAs harbored NSI virus variants during the study.

The group of slow progressors was selected for being clinically and immunologically comparable to the LTAs for 4 years, followed by a decline of their $CD4^+$ cell counts or T cell activity (or both) and development of AIDS within 7 years. Of the 225 seropositive men in follow-up, only 6 met these criteria: 2 were seroconverters (H412 and H450), and the other 4 were seropositive at entry into the cohort. The mean time to the first diagnosis of AIDS was 6.1 ± 1.0 years (table 1). SI viruses were isolated from 2 subjects before AIDS was diagnosed; the other 4 slow progressors persistently harbored NSI viruses.

Subjects were considered rapid progressors on the basis of declining $CD4^+$ cell counts or T cell activity (or both), development of AIDS within 4 years after seroconversion, and the presence of NSI virus variants. Six subjects of all 225 seropositives were considered rapid progressors. They had a mean symptom-free period of 3.2 ± 1.3 years (table 1).

None of the LTAs or slow progressors received any antiviral drugs during the study; however, 3 rapid progressors (H172, H411, and H1145) were treated with zidovudine. H172 started zidovudine treatment at the time of AIDS diagnosis (2.1 years after seroconversion). H411 and H1145 were first treated with antiviral drugs 2.8 and 2.6 years, respectively, after seroconversion. H186, a rapid progressor, was treated with didanosine during the study.

Immunologic parameters. We focused our study on the $CD4^+$ cell counts at 1, 4, and 5 years after seroconversion or entry into the cohort (table 1). Since incidental fluctuation of $CD4^+$ cell counts is frequently seen, the counts were measured as the mean of the number of $CD4^+$ cells present at the visit at the given time point and the visit preceding and following this visit. At year 1, no difference in $CD4^+$ cell counts was seen among the 3 groups. A randomly selected group of seronegative participants of the Amsterdam cohort was tested and showed even lower $CD4^+$ cell counts. The $CD4^+$ cell counts in LTAs showed a slight decline between year 1 and 5, with a mean decline of 25 (± 79) $CD4^+$ cells/μL/year. The slow and rapid progressors showed a substantial decrease of $CD4^+$ cells between year 1 and 5, with a mean of 120 (±67) and 118 (±83) cells/μL/year, respectively, but with different patterns of decline. The rapid progressors showed the strongest decline in $CD4^+$ cell counts in the first years of the study and only a minor decrease between year 4 and 5. The slow progressors showed a gradual decline in $CD4^+$ cell counts between year 1 and 4 but maintained >400 cells/μL. In this group, the strongest decline was observed between years 4 and 5.

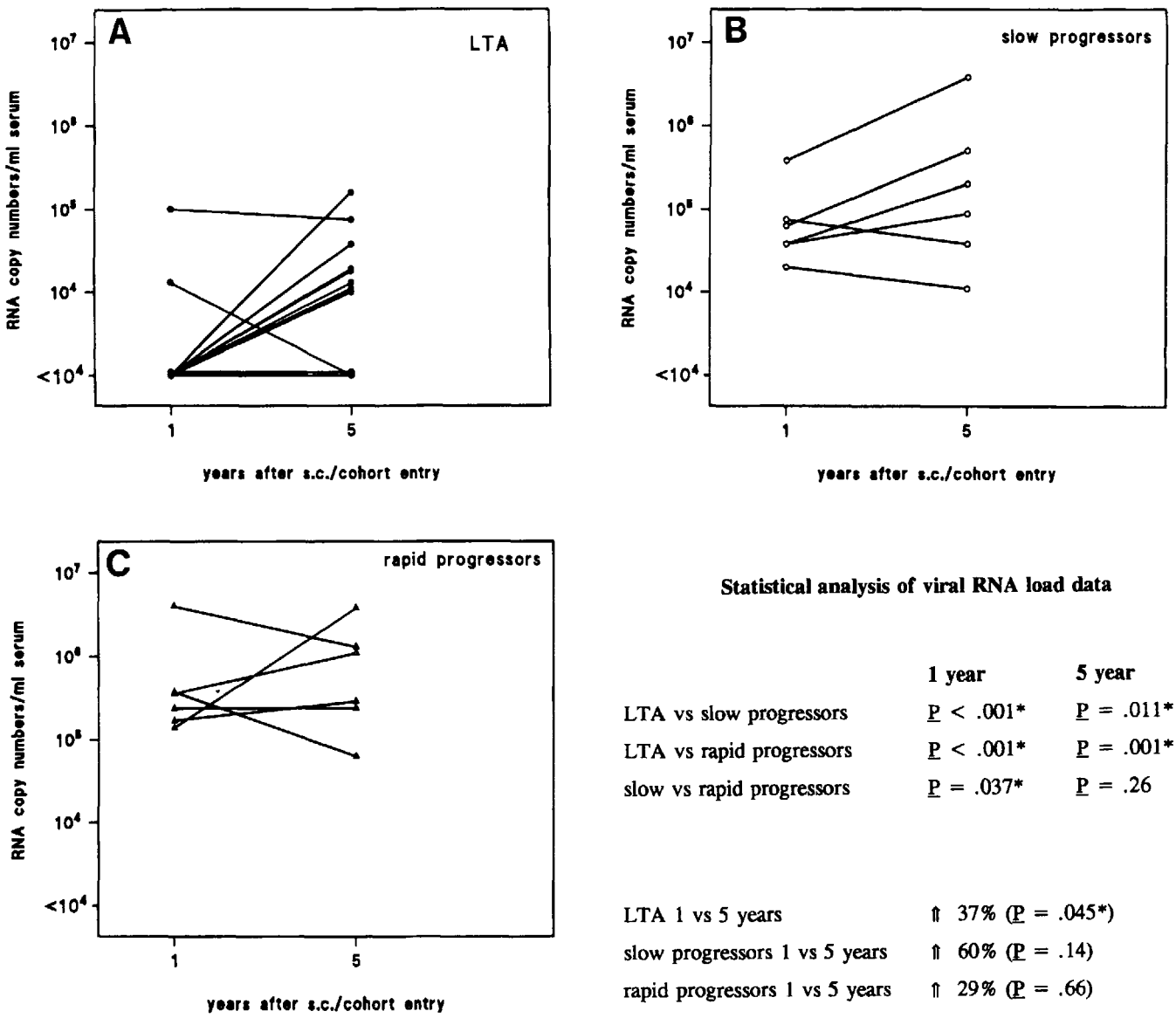
T cell activity, as assessed by anti-CD3 response, was measured to allow differentiation between the groups and was determined at years 4 and 5. Responses >1000 cpm were considered normal [36], although this did not exclude that T helper responses to other stimuli could be diminished [37]. In the LTAs, T cell activity was comparable to that of the seronegative controls (table 1); however, T cell activity in progressors was significantly lower than that in LTAs. T cell activity declined more rapidly in rapid than in slow progressors (table 1). Unfortunately, we were not able to determine the anti-CD3 response at year 1, but results from other studies demonstrated no differences in T cell activity early after infection in progressors and nonprogressors (Roos MT, personal communication).

Viral RNA load in LTAs and progressors. To study differences between LTAs and progressors, we investigated the viral RNA load present in serum from these subjects. We focused on two time points: 1 and 5 years after seroconversion or entry into the cohort. From 1 rapid progressor (H411), the late time-point sample was taken 4 years after seroconversion because this patient died of AIDS within 4.5 years.

HIV-1 RNA load in serum was determined by nucleic acid sequence–based amplification, an assay with a detection level of 10^4 RNA copy numbers/mL serum. At 1 year, 9 of 11 LTAs had an RNA load below the detection level (figure 1A); at 5 years, RNA could still not be detected in 2 of the 9. Patient H211 had a virus load just above the level of detection at 1 year that decreased below this level at year 5. At year 1, the median viral RNA load of the LTAs was $<10^4$ RNA copies/mL (range, $<10^4$–10^5; figure 1A). In slow and rapid progressors, the median viral RNA load was $10^{4.71}$ (range, $10^{4.30}$–$10^{5.58}$) and $10^{5.48}$ RNA copies/mL (range, $10^{5.15}$–$10^{6.60}$), respectively (figure 1B, C). These results showed that the viral RNA load in LTAs at 1 year was significantly lower than in slow and rapid progressors ($P < .001$ for both). A significant difference was also seen between slow and rapid progressors at this time point ($P = .037$).

At year 5, significant differences in RNA levels were also seen. The median viral RNA load for LTAs was $10^{4.11}$ RNA copies/mL (range, $<10^{4.00}$–$10^{5.20}$); for slow and rapid progressors it was $10^{5.15}$ (range, $10^{4.04}$–$10^{6.58}$) and $10^{5.85}$ RNA copies/mL (range, $10^{4.80}$–$10^{6.58}$), respectively. The RNA copy numbers for LTAs were significantly different from both rapid ($P = .001$) and slow progressors ($P = .011$). A comparison of the RNA load early and late in infection showed no significant increase among slow (60% increase, $P = .14$) and rapid (29% increase, $P = .66$) progressors. However, a significant increase in virus load (37%, $P = .045$) was found in LTAs.

Among the slow progressors, 1 subject (H412) had a relatively higher RNA load than the others. Although H412 met the criteria for being a slow progressor, a strong decline in $CD4^+$ cell counts from year 2 to 3 (1000 to 500 cells/μL) was observed, although these counts were never <400 cells/μL during this period. In addition, H412 showed the first symp-



Statistical analysis of viral RNA load data

	1 year	5 year
LTA vs slow progressors	P < .001*	P = .011*
LTA vs rapid progressors	P < .001*	P = .001*
slow vs rapid progressors	P = .037*	P = .26

LTA 1 vs 5 years	⇑ 37% (P = .045*)
slow progressors 1 vs 5 years	⇑ 60% (P = .14)
rapid progressors 1 vs 5 years	⇑ 29% (P = .66)

Figure 1. RNA copy nos. in serum of long-term asymptomatic (LTA; **A**) HIV-infected subjects and those slowly (**B**) and rapidly (**C**) progressing to AIDS. RNA copy nos. were determined 1 and 5 years after seroconversion or cohort entry by quantitative nucleic acid sequence–based amplification. Statistical analysis was done using Mann-Whitney test for comparing different study groups. Paired Student's *t* test using geometric means was used for comparison of data obtained at different time points within 1 study group. *, statistically significant; s.c., seroconversion.

toms of AIDS after 4.7 years, which was the shortest period of progression to AIDS of all slow progressors. Because we knew the time of H412's seroconversion, we concluded that he was probably a rapid progressor even though he met the entry criteria for slow progressors.

The relationship between RNA copy numbers and $CD4^+$ cell count decline per year showed that high $CD4^+$ cell decline was related to high viral RNA copy numbers early in infection (Spearman's correlation coefficient, −0.4, $P = .07$; figure 2).

Individual differences in virus load and the relationship to clinical status were independent of the biologic phenotypes of the viruses harbored by the patients, since all subjects (except for 2 slow progressors) had NSI viruses during the study (table 1).

Patient H8 had the highest viral RNA load among the LTAs and seemed to be the first LTA with indications for disease progression. SI virus variants were detected in this subject in November 1992, 8 years after study entry. In August 1993, 8.8 years after cohort entry, $CD4^+$ cell counts had declined to <400/μL; however, no signs of AIDS were reported in this patient until May 1994. Besides H8, LTA

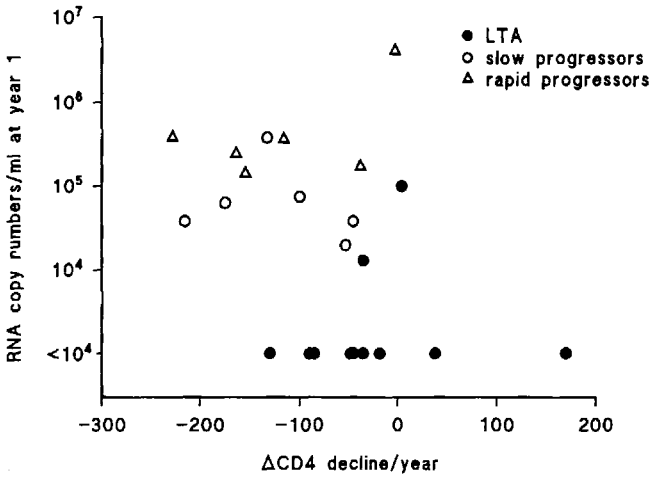


Figure 2. Relationship between RNA copy numbers and $CD4^+$ cell decline. To avoid incidental fluctuation, given $CD4^+$ cell counts are mean counts present at visit at given time point and visit preceding and following that visit. The $CD4^+$ cell decline/year is ratio of difference in cell counts and time studied (4 years). LTA, long-term asymptomatic HIV-infected persons.

patient H157 also developed SI virus variants 8 years after entry into the cohort.

p24 antibody titers and disease progression. The p24 antibody end-point titers in serum of LTAs and progressors were determined to correlate antibody titers to disease resistance (figure 3). As previously shown by Sheppard et al. [38], p24 antibody titers are extremely variable among HIV-infected persons. LTAs could be divided in 2 groups: 1 (n = 6) with low and 1 (n = 5) with higher p24 antibody titers (figure 3A). A nonsignificant increase in p24 antibody titer (45%, P = .24) was seen during follow-up. No significant difference was found between LTAs and slow progressors at 1 year (P = .12) and 5 years (P = .88) because the slow progressors could also be divided into 2 groups: 1 (n = 3) with relatively low but stable p24 antibody titers and 1 (n = 3) with high but declining titers (figure 3B). A nonsignificant decline was seen in the whole group of slow progressors (49%, P = .30). Slow progressor H412, who (as described previously) resembled rapid progressors more than slow progressors, had the lowest p24 antibody end-point titer. This result matched with the finding that the group of rapid progressors all had extremely low p24 antibody end-point titers (figure 3C).

A significant decline in p24 antibody titer (91%, P = .007) was seen over time in the rapid progressors. The p24 antibody titers of slow and rapid progressors were not significantly different at 1 year (P = .07) but were at 5 years (P = .005). In addition, the antibody titers of LTAs at 5 years were significantly higher than those of rapid progressors (P = .001).

Relationship between viral RNA load and p24 antibody titers. In figure 4 the relationship between p24 antibody titers and viral RNA load in serum at 1 year is shown. No significant correlation was found between p24 antibody titer and disease progression (Fisher's exact test, P = .10). However, in subjects with a detectable virus load, the relative risk for rapid progression to AIDS in the presence of low p24 antibody titers was 5.0 (95% confidence interval, 0.77–32.6). This means that the risk to develop AIDS rapidly is 5.0 times higher in persons with low p24 antibody titers than in those with high p24 antibody titers.

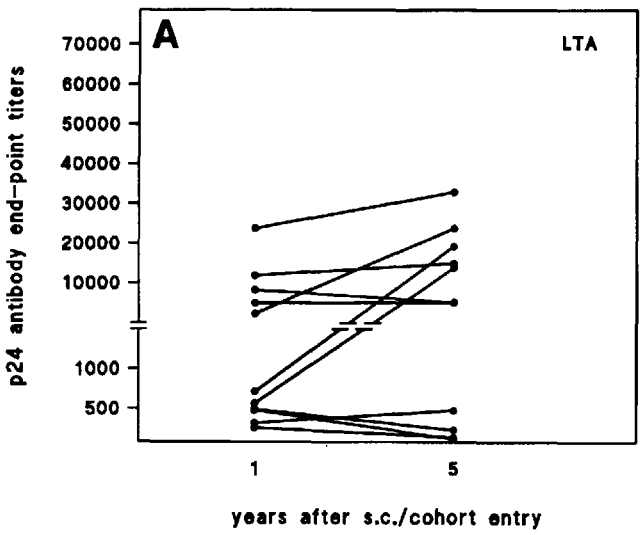
Reactivity to V3 and gp41 peptides of sera from LTAs and progressors. To study the role of envelope-specific antibodies in disease progression, we tested early and late sera for reactivity to a set of well-defined peptides covering the V3 domain of HIV-1. The peptides were based on known sequences of MN-like viruses circulating most prevalently in Europe and the United States, as described by LaRosa et al. [39], and on sequences of viruses in HIV-1–infected Africans, as described by Zwart et al. [32] (table 2). The sera were also tested for reactivity to a peptide derived from a constant domain of gp41. The set of peptides was previously used to study the serum reactivity of participants of the Amsterdam cohort: Zwart et al. [31] showed that 85% of the cohort predominantly showed reactivity to p108, p109, or p110, or a combination of these 3 peptides, which differ at only one amino acid position. The results of our study confirmed these data: no difference was observed among LTAs or progressors in peptide specificity, and in time, a broadening of the reactivity was seen in all 3 groups (data not shown).

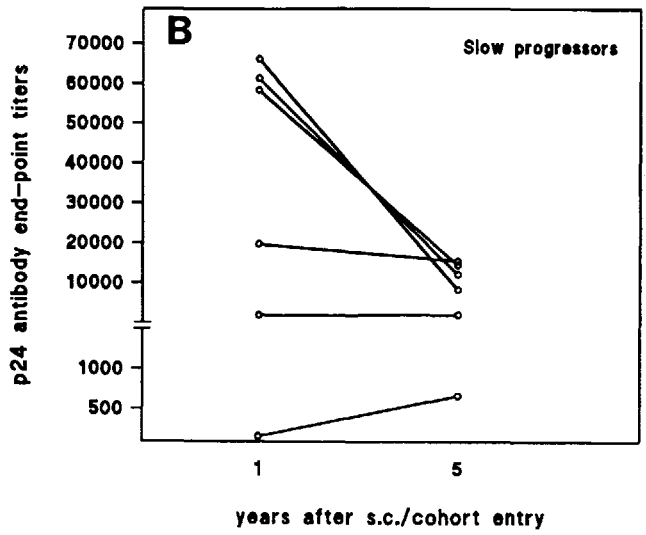
If specificity to a certain peptide was found, the antibody end-point titer to the peptide was determined. The mean end-point titers to the peptide with the highest reactivity and to the gp41 peptide are shown in figure 5. No significant differences among the groups were found at 1 and 5 years, mainly because of high SDs. However, end-point titers of the rapid progressors, both to V3 peptides and the gp41 peptide, tended to be lower.

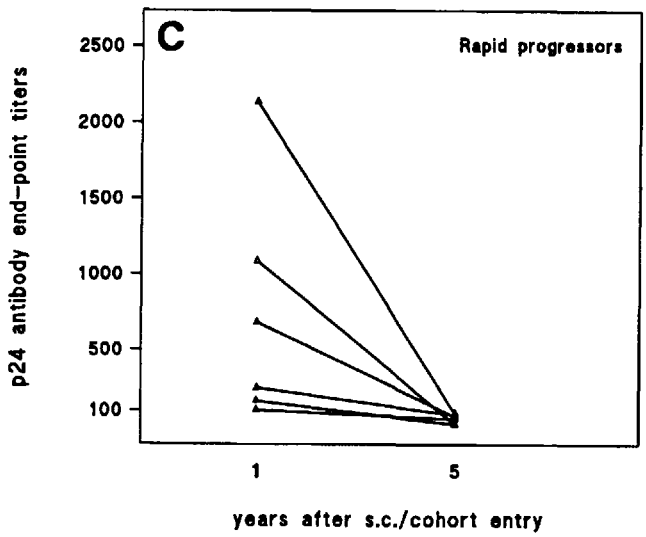
Neutralization of laboratory virus strains by sera from LTAs and progressors. To investigate whether differences in viral RNA load were due to distinctions in neutralizing properties of sera from LTAs and progressors, we tested early and late sera from both groups for neutralization of laboratory strains MN, HXB3, and RF. The reciprocal neutralization titers to MN are shown in figure 6. An increase in neutralization titer over time was seen in LTAs and progressors, but no significant difference was found between the 3 groups. Low neutralizing capacities were seen to HXB3 and RF in sera with high neutralizing titers to MN, indicating cross-reactivity of these sera (data not shown).

Discussion

We selected groups of LTAs and progressors from the Amsterdam cohort to obtain data that contribute to the understanding of the pathogenesis of HIV-1 infection. Due to our







Statistical analysis of p24 antibody titers

	1 year	5 year
LTA vs slow progressors	P = .12	P = .88
LTA vs rapid progressors	P = .12	P = .001*
slow vs rapid progressors	P = .07	P = .005*
LTA 1 vs 5 years	⇑ 45% (P = .24)	
slow progressors 1 vs 5 years	⇓ 49% (P = .30)	
rapid progressors 1 vs 5 years	⇓ 91% (P = .007*)	

Figure 3. p24 antibody titers in long-term asymptomatic (LTA; **A**) HIV-infected subjects and those slowly (**B**) and rapidly (**C**) progressing to AIDS. Titers determined by ELISA 1 and 5 years after seroconversion or cohort entry. Statistical analysis was done using Mann-Whitney test for comparing different study groups. Paired Student's *t* test using geometric means was used for comparison of data obtained at different time points within 1 study group. *, statistically significant; s.c., seroconversion.

stringent selection criteria, we acquired limited but well-defined groups of LTAs and progressors representing a smaller proportion of the population than those described in other papers [19, 40, 41]. The study groups included seroconverters and seropositive subjects with unknown seroconversion dates. However, there are two reasons to assume that the latter had been infected shortly before cohort entry: first, HIV infection was introduced in The Netherlands in the early 1980s, with a maximum incidence in 1984 [42]; second, using the Kaplan-Meier product limit method, progression to AIDS did not differ between men who were seropositive at entry and those with a known time of seroconversion [40].

Since longitudinal serum samples were available from all participants, we determined viral RNA load at two time points: 1 and 5 years after seroconversion or entry in the cohort. We chose to study viral RNA load in serum because evidence has been obtained by our group that it is comparable to that in plasma (unpublished data) and because RNA load in plasma was shown to be a more sensitive index for changes in HIV-1 activity than HIV-1 proviral copy numbers in PBMC [43].

At 1 year, LTAs and progressors could not be discriminated serologically or immunologically; however, we found a striking difference in serum RNA load between these groups, from which we concluded that the initial RNA load is predic-

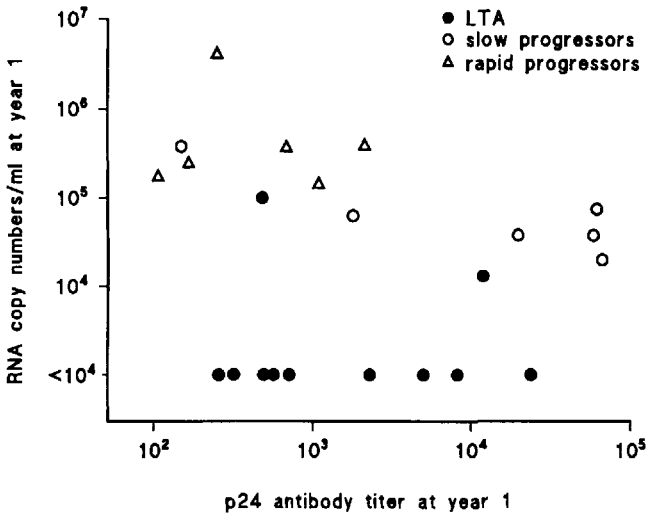


Figure 4. Correlation between RNA copy number and p24 antibody titer early in HIV-1 infection. LTA, long-term asymptomatic HIV-infected subjects.

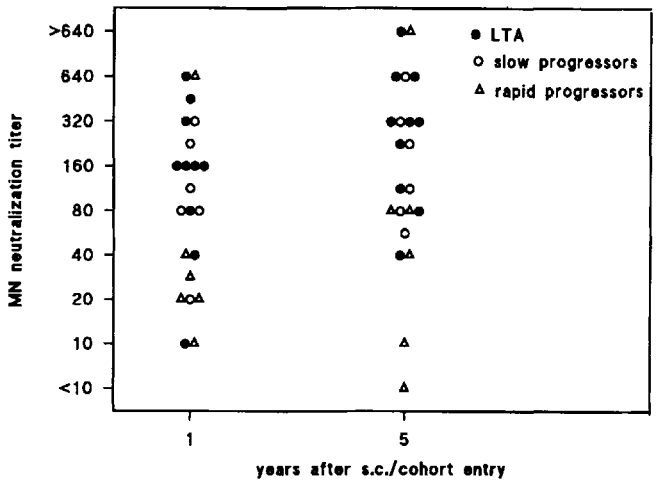


Figure 6. MN neutralization titers of long-term asymptomatic (LTA) HIV-infected subjects and those slowly and rapidly progressing to AIDS. Shown are reciprocal serum dilutions resulting in 99% inhibition of syncytium formation. Statistical analysis was done using Mann-Whitney test. s.c., seroconversion.

tive of clinical outcome. In addition, within the groups of progressors, no significant differences were seen in viral RNA load at 1 and 5 years. An increase in serum RNA was found in 7 of 11 LTAs at 5 years, suggesting that these subjects also will develop AIDS eventually. The finding that rapid progressors retain high HIV-1 RNA levels throughout the symptom-free period is supported by several other studies [43–45]. A recent report by Lee et al. [46] showed that the DNA load in PBMC is relatively stable over time within each person but varies greatly among subjects [46]. Bagnarelli et al. [43] demonstrated that both HIV-1 DNA in PBMC and HIV-1 RNA in plasma were virtually stable in sequential samples from clinically stable, asymptomatic subjects. On the other hand, Connor et al. [16] described a stable DNA load in PBMC of an LTA and increasing DNA burden in progressors, correlating with the appearance of viruses with the SI phenotype.

Since our study groups were selected on the basis of $CD4^+$ cell counts, we can compare our study to that of Sheppard et al. [19], who followed a group of HIV-1 infected persons with different rates of $CD4^+$ cell loss and progression to AIDS for up to 78 months. A more pronounced viremia, measured by the frequency of virus recovery from HIV-1 isolation cultures, was seen in subjects with a rapid $CD4^+$ cell decline and was associated with the presence of SI viruses. The prognostic value of the presence of SI viruses and progression to AIDS was previously demonstrated [7–9, 47–49]. In our study, to exclude differences in viral replication caused by more virulent viruses, all LTAs and all but 2 progressors were selected because they harbored NSI viruses at study entry and during follow-up. This demonstrates that, as previously reported [8, 19], progression to and death from AIDS without a virus phenotype conversion is frequently seen. Two slow progressors (H222, H450) switched from NSI to SI during follow-up. The virus phenotype of H222 was tested once a year, and the first known SI virus isolate was found 2.5 years after cohort entry. His viral RNA load at 1 and 5 years increased almost 10-fold at the time the phenotype switch was seen. Of interest, in agreement with the findings of Connor et al. [16], the first SI isolate of H450 was found 5 years after seroconversion and was associated with a

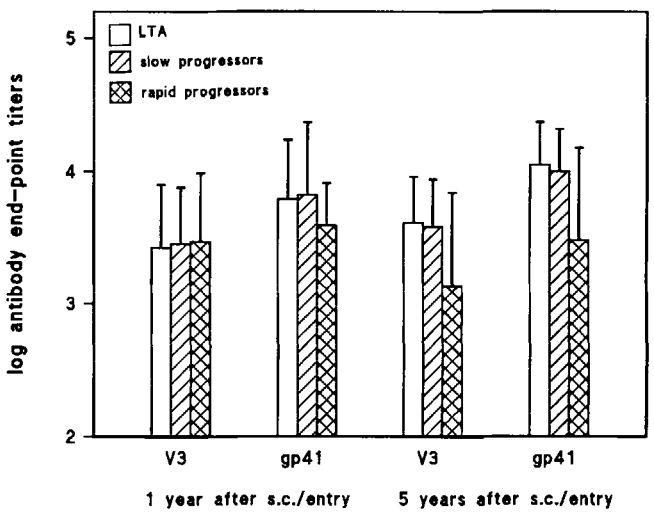


Figure 5. Geometric mean antibody end-point titers to V3 and gp41 peptides in long-term asymptomatic (LTA) HIV-infected subjects and those slowly and rapidly progressing to AIDS. Antibody titers were determined using ELISA. Statistical analysis was done using Student's *t* test for geometric means. s.c., seroconversion.

decline in $CD4^+$ cell count to <400 cells/μL and an increasing virus burden.

Virus burden after primary infection is the result of numerous host and virus factors. In the host, the humoral and cellular immune response to the virus are crucial, and the initial immune response to HIV and immune system activation is suggested to determine the outcome of AIDS [38]. Humoral immune responses are reflected in the amount of antibodies to various HIV-1 antigens, such as p24, p17, or gp120, or by the presence of neutralizing antibodies. To study the role of the envelope-specific humoral immune response, all subjects were tested for the presence of V3- and gp41-specific antibodies and neutralizing antibodies to laboratory strains. In addition, p24 core antibody end-point titers were determined.

Our group previously showed that the specificity of the early antibody response accurately reflects the virus population circulating at the time of seroconversion [31]. The early response appeared to be preserved, and changes in serum specificity at a later stage were rare, since specific responses to variants emerging at later stages were absent or of low level. From these results, we concluded that all subjects were infected with virus variants related to the MN strain, since no major differences were found in peptide specificity. No distinctions were found in the V3 antibody end-point titers among the different groups at 1 or 5 years, indicating that the humoral immune response to envelope V3 peptides is not related to disease progression.

Another study on V3 peptide reactivity in progressing and nonprogressing seroconverters, who were selected only for the length of the disease-free period, demonstrated a significant elevation in V3 peptide mid-point titer at 3 months after seroconversion in progressors and a delay in V3 antibody development in the nonprogressing group [50]. These differences could not be seen in the present study because sera were tested at 1 and 5 years only. From our data, no relationship was found between the presence of V3-specific antibodies and disease resistance. In addition, in a report on heterosexual transmission, no difference in V3-specific antibodies was found between transmitting and nontransmitting persons [51].

Antibodies to the core proteins p24 and p17 have been shown to be of prognostic significance, since a drop in serum antibodies against these proteins was shown to be correlated with progression to AIDS [1, 40, 52–54]. This is supported by our finding that the lowest p24 antibody levels were found in the group of rapid progressors. Two explanations can be given for the low levels: higher virus burden, which results in complex formation, and low antibody titers due to impairment of the mechanism of antibody production in rapid progressors.

Teeuwsen et al. [55] showed that the decline in p24-specific antibodies, which is observed during progression to AIDS, is not merely a reflection of the clearance via immune complexes, but may also be partly attributed to a reduction of functionally active B lymphocytes producing p24-specific antibodies. This was recently confirmed by Fenouillet et al. [56], who showed that acid dissociation of complexes did not increase p24 antibody levels. Furthermore, in a study of vertical transmission, high anti-p24 levels correlated with a low risk of transmission, whereas low anti-p24 titers were associated with an increased risk of vertical transmission [57]. In 5 of 11 LTAs, low virus load was associated with low p24 antibody titers. Possibly, this can be explained by the fact that the number of circulating virus particles in these subjects was not sufficient to elicit a high antibody response, while in the other subjects, the virus load, although still below the RNA detection level, was enough to raise a high p24 antibody response.

The immune system may be able to clear viruses or prevent them from spreading by generating neutralizing antibodies. Although the occurrence of neutralizing antibodies in serum of HIV-1 infected persons was first described in 1985 by Robert-Guroff et al. [58] and Weiss et al. [59], their role in prevention, natural course, and progression of HIV infection has not yet been elucidated. Some longitudinal studies showed protection by neutralizing antibodies because of a correlation between their presence and a better clinical outcome [19, 60–64]. However, data have also been reported that showed no correlation between the presence of neutralizing antibodies and development of disease [65–68].

From our experiments, we conclude that antibodies neutralizing laboratory strains MN, RF, and HXB3 are not involved in disease protection. Our data can be compared with those of Lifson et al. [17], who reported that no significant difference was found in neutralization of laboratory strain IIIB in nonprogressors with $CD4^+$ cell counts >400 cells/μL, although no information is given about the time point at which the serum was obtained from the patients. However, no neutralization data were obtained using primary isolates or autologous virus. This is presently under investigation. Recently obtained results from Lu et al. [69], who did a 2-year follow-up of patients with different disease progression rates, showed that persons rapidly progressing to AIDS with a high rate of $CD4^+$ cell decline had significantly higher concentrations of infected PBMC and showed low neutralizing activity of autologous virus. Preliminary results of Ho and coworkers showed that primary isolates could be better neutralized by sera from LTAs than by sera from progressors (Ho DD, personal communication).

In conclusion, our results show that early in HIV-1 infection, the number of viral RNA copies in serum is predictive of the clinical course of disease. LTAs were shown to have low or undetectable levels of viral RNA, whereas progressors had substantial amounts of viral RNA in their sera. Moreover, progressors with a high virus load and high levels of p24 antibodies had a 5 times lower relative risk for rapid progression to AIDS than progressors with high virus load

and low p24 antibodies. This indicates that despite a high viral RNA load early in infection, the delay of progression to AIDS is related to the presence of p24-specific antibodies.

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Decline in the AIDS and death rates in the EuroSIDA study: an observational study

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Background Since the introduction of highly active antiretroviral therapy (HAART), little is known about whether changes in HIV-1 mortality and morbidity rates have been sustained. We aimed to assess possible changes in these rates across Europe.

Methods We analysed data for 9803 patients in 70 European HIV centres including ones in Israel and Argentina. Incidence rates of AIDS or death were calculated for overall and most recent CD4 count in 6-monthly periods and in three treatment eras (pre-HAART, 1994–1995; early-HAART, 1996–1997; and late-HAART, 1998–2002).

Findings The incidence of AIDS or death fell after September, 1998, by 8% per 6-month period (rate ratio 0·92, 95% CI 0·88–0·95, p<0·0001). When AIDS and death were analysed separately, the incidence of all deaths during the late-HAART era was significantly lower than that during the early-HAART era in patients whose latest CD4 count was 20 cells/μL or less (0·43, 0·35–0·53, p<0·0001), but at higher CD4 counts, did not differ between early-HAART and late-HAART. Incidence of AIDS was about 50% lower in late-HAART than in early-HAART, irrespective of latest CD4 count (p<0·0001). In multivariate Cox's models, with early-HAART as the reference, there was an increased risk of AIDS (relative hazard 1·39; 95% CI 1·16–1·67, p=0·0004) and all deaths (1·29; 1·08–1·56, p=0·0065) in the pre-HAART era, and a reduced risk of AIDS (0·62; 0·50–0·77, p<0·0001) and all deaths (0·66; 0·53–0·82, p=0·0002) in the late-HAART era.

Interpretation The initial drop in mortality and morbidity after the introduction of HAART has been sustained. Potential long-term adverse effects associated with HAART have not altered its effectiveness in treating AIDS.

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*For list of members see
http://image.thelancet.com/extras/02art10332webappendix.pdf

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Introduction

The introduction of highly active antiretroviral therapy (HAART) during 1996 and 1997 led to a well-documented reduction in mortality and risk of AIDS-defining illnesses.[1–4] The death rate across Europe dropped rapidly, and within 2 years of the widespread availability of HAART,[5] the number of deaths were less than a fifth of those before HAART.[1] The initial success associated with HAART might not have continued, and high levels of treatment failure have been reported,[6–8] which has been associated with serious adverse events, emergence of drug resistance, difficulties in maintenance of long-term adherence, and the few types of drugs available.[9–10]

The EuroSIDA study was started in 1994 and provides an ideal opportunity to follow a large cohort of patients to describe patterns of mortality and morbidity in the era of HAART. The aims of this study were to examine changes in AIDS and death rates during this time.

Methods

Patients

The EuroSIDA study is a prospective study of patients with HIV-1 in 70 centres across Europe, and now includes Argentina and Israel.[11] The centres provided data for consecutive patients seen in outpatient clinics from May, 1994, until a predefined number of patients was enrolled from each centre. To be eligible for inclusion, patients needed to have a prebooked clinic appointment and be aged over 16. The EuroSIDA I cohort was the first 3116 patients enrolled. The second cohort (n=1365) was enrolled between November, 1995, and April, 1996, the third (n=2839) between February, 1997, and September 1997, the fourth (n=1225) between January, 1999 and December, 1999, and the fifth (n=1258) between November, 2001, and April, 2002. For cohorts I–III, eligible patients were those with a CD4 count less than 500 cells/μL in the previous 4 months at enrolment. The threshold CD4 count was removed for cohorts IV and V. Information from patient notes was provided on a standardised data collection form at baseline and every 6 months thereafter. Follow-up was to September, 2002 with information available from up to 16 forms for cohort I, 13 for cohort II, ten for cohort III, five for cohort IV, and one for cohort V. At every follow-up visit, data were obtained for all CD4 counts measured since last follow-up and for viral load measurements. Height, weight, haemoglobin, and other laboratory indices were also routinely measured.

For all patients, the date of starting and stopping every antiretroviral drug was recorded, as was the use of drugs for prophylaxis against opportunistic infections. Dates of diagnosis of all AIDS-defining illnesses were also recorded, including subsequent diagnoses, by use of the 1993 clinical definition of AIDS from the Centers for Disease Control, USA.[12] Date variables were recorded as

month and year. A rigorous quality assurance programme has been established that includes data control at the coordinating centre, site visits to check patient selection, and cross-checking data provided against patient notes. Information for patients not seen for over 12 months were regularly reviewed to try to obtain date of death, further follow-up information, or details of the clinic they might have moved to.

Informed consent and ethics committee approval were obtained in every participating centre according to national guidelines.

Statistical methods

Three treatment periods were defined before the study; the pre-HAART era (1994–1995), early-HAART (1996–1997), and late-HAART (1998 onwards). The characteristics of patients were analysed separately according to the treatment era in which they were recruited. To calculate event rates, we used 6-month intervals using the same calendar periods and methodology as described previously.[1] The incidence of AIDS or death was calculated as the number of events per person-years of follow-up (PYFU) for every 6-month period. Patients with AIDS at recruitment were followed until diagnosis of their next AIDS-defining illness or death, whichever arose first. Recurrences of AIDS-defining illnesses were excluded from analyses. We calculated the median CD4 count of patients under observation during each 6-month period using the CD4 count closest to the midpoint of the period. As in previous EuroSIDA analyses,[1] follow-up between recruitment and first follow-up was excluded because of the possible selection bias of very late stage patients not being included in the study.[1] Trends over time were investigated with Poisson regression and the likelihood ratio test. Additionally, Cox's proportional hazards models were used to estimate the relative hazard of AIDS or death.[1] Every 6-month period was fitted as a time-dependent covariate to test whether risk of AIDS or death changed over time.

Within each treatment era the incidence of AIDS, all deaths, and HIV-1 related deaths was calculated separately and according to the current CD4 count. Cox's proportional hazards models were used to calculate the relative hazard of AIDS, all deaths and HIV-1 related deaths in different treatment eras with three different models. The models were (1) a univariate model, (2) a multivariate model adjusted for known factors at recruitment (CD4 count, age, previous treatment, whether a patient had started HAART or not, previous AIDS diagnoses and in a subgroup analysis, viral load), and (3) a model that adjusted for the CD4 count as a time-dependent covariate to account for changes in the CD4 count over follow-up. Poisson regression analyses were done with STATA (version 7), and all other analyses were done with SAS (version 6.12).

	All n (%)	Pre–HAART (1994–1995) n (%)	Early–HAART (1996–1997) n (%)	Late–HAART (1998) n (%)	p*
All	9803 (100%)	3793 (38·7%)	3425 (34·9%)	2585 (26·4%)	
Sex					
Male	7678 (78·3%)	3051 (80·4%)	2670 (78·0%)	1957 (75·7%)	<0·0001
Female	2125 (21·7%)	742 (19·6%)	755 (22·0%)	628 (24·3%)	
Risk group					
Homosexual	4403 (44·9%)	1802 (47·5%)	1515 (44·2%)	1086 (42·0%)	<0·0001
IDU	2405 (24·5%)	1016 (26·8%)	787 (23·3%)	592 (22·9%)	
Heterosexual	2319 (23·7%)	738 (19·5%)	880 (25·7%)	701 (27·1%)	
Other	676 (6·9%)	237 (6·2%)	233 (6·8%)	206 (8·0%)	
Ethnic origin					
White	8398 (85·7%)	3226 (85·1%)	2881 (84·1%)	2291 (88·6%)	<0·0001
Other	1405 (14·3%)	567 (14·9%)	544 (15·9%)	294 (11·4%)	
Region of Europe					
South	2891 (29·5%)	1438 (37·9%)	1043 (30·4%)	410 (15·9%)	<0·0001
Central	2609 (26·1%)	943 (24·9%)	1161 (33·9%)	505 (19·5%)	
North	3191 (32·6%)	1412 (37·2%)	1221 (35·7%)	558 (21·6%)	
East	1012 (10·3%)	0 (··)	0 (··)	1012 (39·2%)	
Argentina	100 (1·0%)	0 (··)	0 (··)	100 (3·9%)	
AIDS before enrolment	2983 (30·4%)	1278 (33·7%)	1044 (30·5%)	661 (25·6%)	<0·0001
ARV history					
ARV naive	2136 (21·8%)	953 (25·1%)	668 (19·5%)	515 (19·9%)	<0·0001
Previous nucleosides	7583 (77·4%)	2782 (73·4%)	2748 (80·2%)	2053 (79·4%)	<0·0001
Previous PI	3019 (30·8%)	60 (1·6%)	1379 (40·3%)	1580 (61·1%)	<0·0001
Previous NN	953 (9·7%)	27 (0·7%)	129 (3·8%)	797 (30·8%)	<0·0001
All 3 classes	526 (5·4%)	2 (0·0%)	55 (1·6%)	409 (18·1%)	<0·0001
Disease specific treatment					
PCP	4515 (46·1%)	2123 (56·0%)	1676 (48·9%)	716 (27·7%)	<0·0001
TB	539 (5·5%)	258 (6·8%)	182 (5·3%)	99 (3·8%)	<0·0001
CMV	498 (5·1%)	235 (6·2%)	163 (4·8%)	100 (3·9%)	<0·0001
Fungals	967 (9·9%)	632 (16·7%)	214 (6·2%)	121 (4·7%)	<0·0001
Laboratory tests					
CD4 (median, IQR)	238 (108–380)	168 (48–302)	223 (112–340)	380 (230–562)	<0·0001
VL (median, IQR)	3·11 (2·30–4·37)	4·37 (3·30–4·99)	3·53 (2·70–4·60)	2·60 (1·70–3·96)	<0·0001
Age (median, IQR)	36·2 (31·3–43·2)	35·3 (30·7–42·6)	36·7 (32·0–43·6)	36·8 (31·2–43·8)	<0·0001

Percentages are shown in parentheses, unless otherwise indicated. Disease specific treatment refers to the number and proportion of patients who had ever used specific drugs at recruitment to EuroSIDA. See EuroSIDA follow-up form for details of drugs included in each category.[13] Viral load was measured at or before enrolment for 5169 (52·7%) patients; 154 (4·1%), 2600 (75·9%) and 2415 (93·4%) for the pre-HAART, early-HAART, and late-HAART eras, respectively. IDU=intravenous drug user. ARV=antiretroviral. PI=protease inhibitors. NN=non-nucleosides. PCP=*P carinii* pneumonia. TB=tuberculosis. CMV=cytomegalovirus. VL=viral load. *p values were obtained from a χ^2 test for categorical variables and Wilcoxon test for continuous variables.

Table 1: **Characteristic of patients at recruitment to EuroSIDA during three treatment eras**

Tests for differences between categorical variable were done with χ^2 tests, and differences in continuous variables were tested with the Wilcoxon test.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Table 1 shows characteristics of the patients grouped according to whether they were enrolled pre-HAART, early-HAART, or late-HAART. As expected, demographic characteristics of patients in the various treatment eras differed greatly. The proportion of intravenous drug users enrolled fell over time, from 26·8% in the pre-HAART era to 22·9% in late-HAART ($p<0·0001$). Because of the study design, no patients were enrolled from Eastern Europe or Argentina until the late-HAART era. Patients recruited in this period were significantly less likely to have AIDS at enrolment ($p<0·0001$) and also had much higher CD4 counts at enrolment than those recruited in the early eras ($p<0·0001$). At enrolment, the proportion of patients who had not previously been given antiretrovirals decreased ($p<0·0001$), and the proportion who, at enrolment, had previously been treated with nucleosides, protease inhibitors, or non-nucleosides, or all three, increased (all groups $p<0·0001$). Additionally, the proportion of patients using disease specific drugs, including prophylaxis for *Pneumocystis carinii* pneumonia and antifungal drugs fell (both $p<0·0001$).

Figure 1 illustrates the changes over time in the CD4 count of patients under follow-up; the median CD4 count at the mid-point of each period rose from 164 cells/μL, (interquartile range [IQR] 40–300 cells/μL) during September, 1994, to March, 1995, by about 10–30 cells/μL per 6-month period, to 424 cells/μL (270–600 cells/μL) in patients under follow-up after September, 2001. Over the same period, the percentage of patients with a CD4 count of 50 cells/μL or less dropped from 28·0% to 2·6%, whereas the percentage of those with a CD4 count higher than 200 cells/μL rose from 42·2% to 84·5%.

Figure 2 shows the change in the combined AIDS and death rate (events) over time with the median CD4 count at the event. Between September, 1994, and March, 1995, the event rate was 43·5 per 100 PYFU (95% CI 39·8–47·2 per 100 PYFU); this rate steadily fell over time and by March, 1998, to September, 1998, it was less than 5 per 100 PYFU, which is about a tenth of that at the start of the investigation. Since September, 1998, the event rate has continued to fall. A similar pattern was seen within each cohort (data not shown). Using poisson regression, we recorded a significant reduction in the event rate since September, 1998. This rate dropped by an estimated 8% with every additional 6-month period (rate ratio [RR] 0·92; 95% CI 0·88–0·95, $p<0·0001$). Over the 8 years of the study, we noted a pronounced and steady increase in the median CD4 count at the time of the event—from 29 cells/μL to 224 cells/μL.

The reduction in AIDS or death rates could be partly explained by the results of figure 1, which showed a substantial rise in CD4 count over time, attributable to the increased use of HAART in EuroSIDA patients, and the addition of new cohorts with higher CD4 counts. Figure 3 shows the relative risk of AIDS or death of patients under follow-up in every 6-month period, after adjustment for

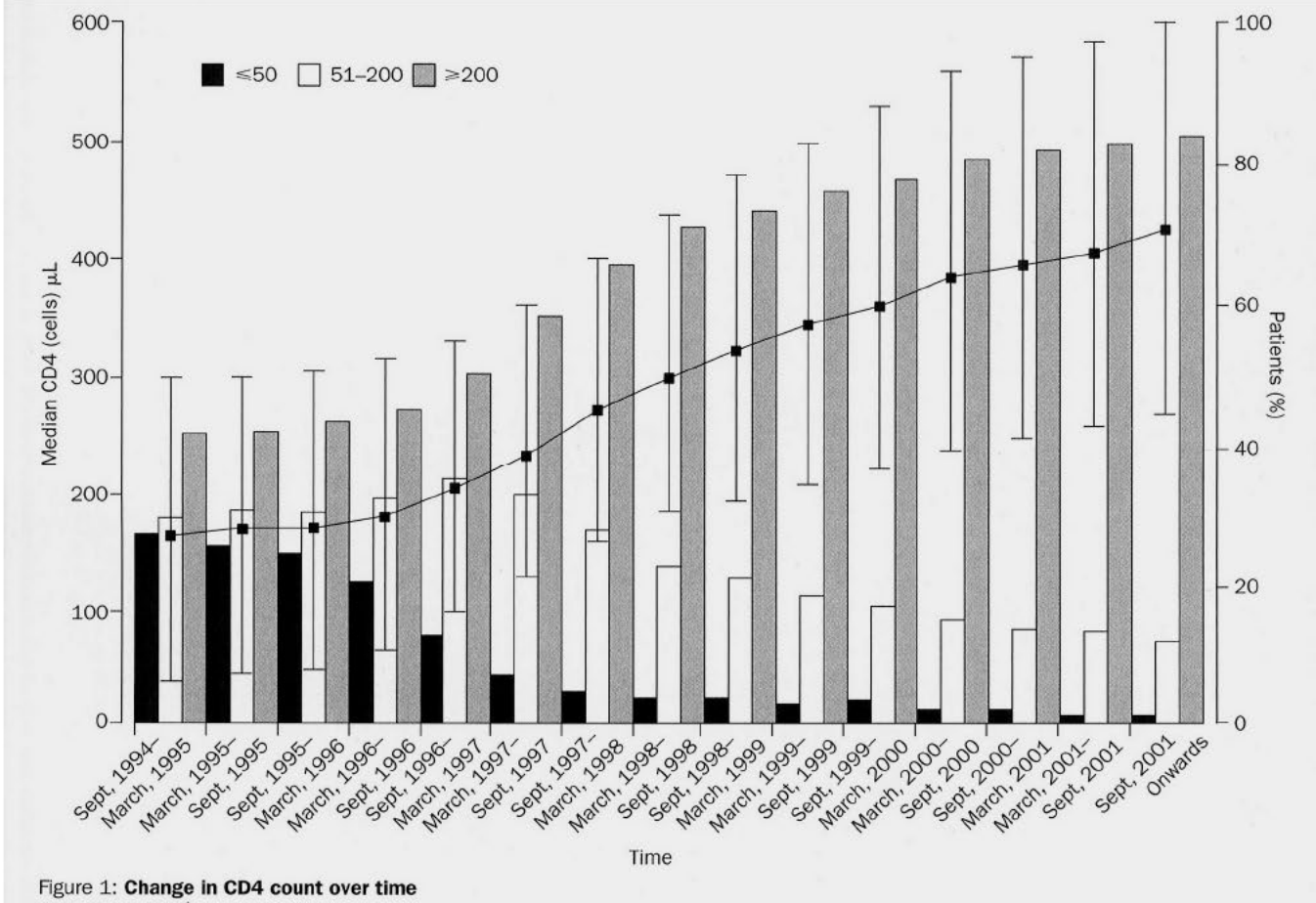

Figure 1: **Change in CD4 count over time**
Vertical bars=IQR.[7]

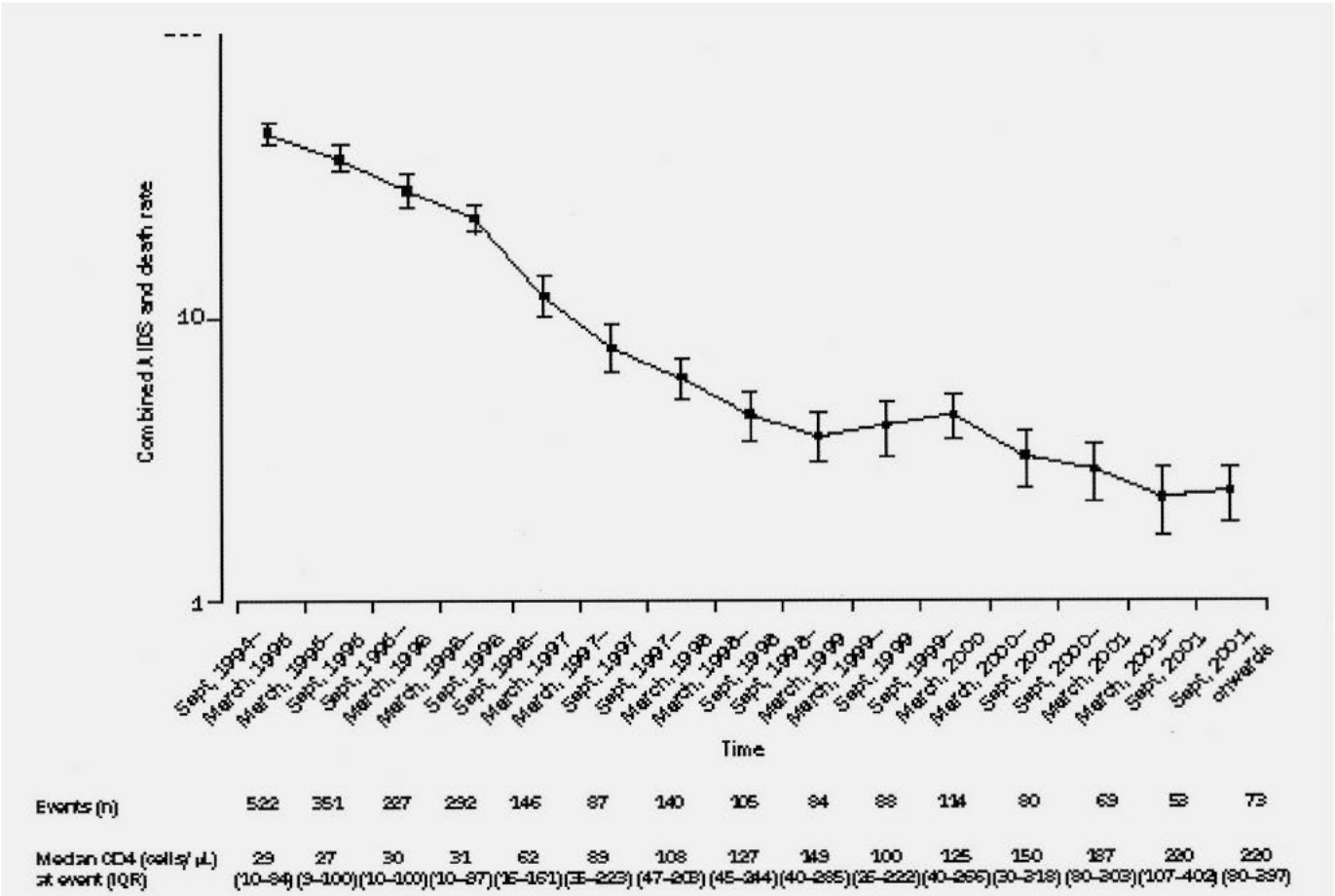

Figure 2: **Combined AIDS and death rates**
Vertical bars=95% CIs.

CD4 count at recruitment to the study, age, AIDS status, and whether the patient had started HAART at recruitment or not. September, 1998–March, 1999, was used as the reference period. After adjustment, there remains a significant reduction in the relative risk of AIDS or death in every 6-month period until the period March–September, 1997. During the following 3 years the relative risk stayed around 1, but patients under follow-up during March–September, 2001, were at a significantly reduced risk of AIDS or death (relative hazard [RH] 0·73; 95% CI 0·54–0·98, p=0·038), as were patients under follow-up after September, 2001 (RH 0·72, [0·56–0·93], p=0·013). Viral load was not routinely measured in clinics until 1997, and these data were not available at baseline for almost 50% of the patients. However, a further analysis that adjusted for viral load at recruitment showed similar results (data not shown), as did analyses that adjusted for disease-specific prophylaxis, other markers of disease progression (such as body-mass index), and pre-HAART antiretroviral treatment.

Table 2 shows the change in the incidence of AIDS or death, according to latest CD4 count and treatment era. This method of analysis takes account of changes in the CD4 count, and PYFU accrue as patients move within categories. Thus, patients with similar current CD4 counts were compared across treatment eras, irrespective of cohort. Within every CD4 count strata, median CD4 count across treatment periods changed little (data not shown). The exception to this finding was a significant increase in patients with CD4 counts greater than 350 cells/μL. In the pre-HAART and early-HAART eras, the median CD4 count was 430 cells/μL (IQR 388–516 cells/μL), whereas in the late-HAART era this rose to 515 cells/μL (418–640 cells/μL).

The incidence of all deaths has fallen from the pre-HAART era to late-HAART across all CD4 count strata. For example, in patients whose latest CD4 count was 20 cells/μL or less, the incidence of all deaths dropped from 68·9 per 100 PYFU in the pre-HAART era to 34·6 in the late-HAART era. However, in those whose latest CD4 count was between 21–350 cells/μL, little improvement in the overall death rate was seen on comparison of the early-HAART and late-HAART eras. In patients with CD4 counts of 20 cells/μL or less, the incidence of all deaths in the late-HAART era was less than half that during the early-HAART era (RR 0·43, 95% CI 0·35–0·53, p<0·0001). There was also a significant reduction in the incidence of all death in patients with CD4 counts higher than 350 cells/μL (0·59, 0·35–0·98, p=0·041), although notably this group had a high median CD4 count. A similar decline in incidence from the pre-HAART to late-HAART across all CD4 counts was seen for HIV-1 related deaths. Furthermore, patients with low CD4 counts (≤50 cells/μL) and those with high CD4 counts (≥200 cells/μL) showed a drop in the incidence of HIV-1 related deaths in comparisons of early–HAART and late-HAART treatment eras. Patients with intermediate CD4 counts (51–200 cells/μL) had no further reduction in the incidence of deaths between these periods.

The change in AIDS rates showed a different pattern from that of deaths (table 2). In general, the incidence of AIDS in the late-HAART era was between a quarter and a half of that in the pre-HAART era, irrespective of CD4 count. Additionally, there was a strong decrease from the

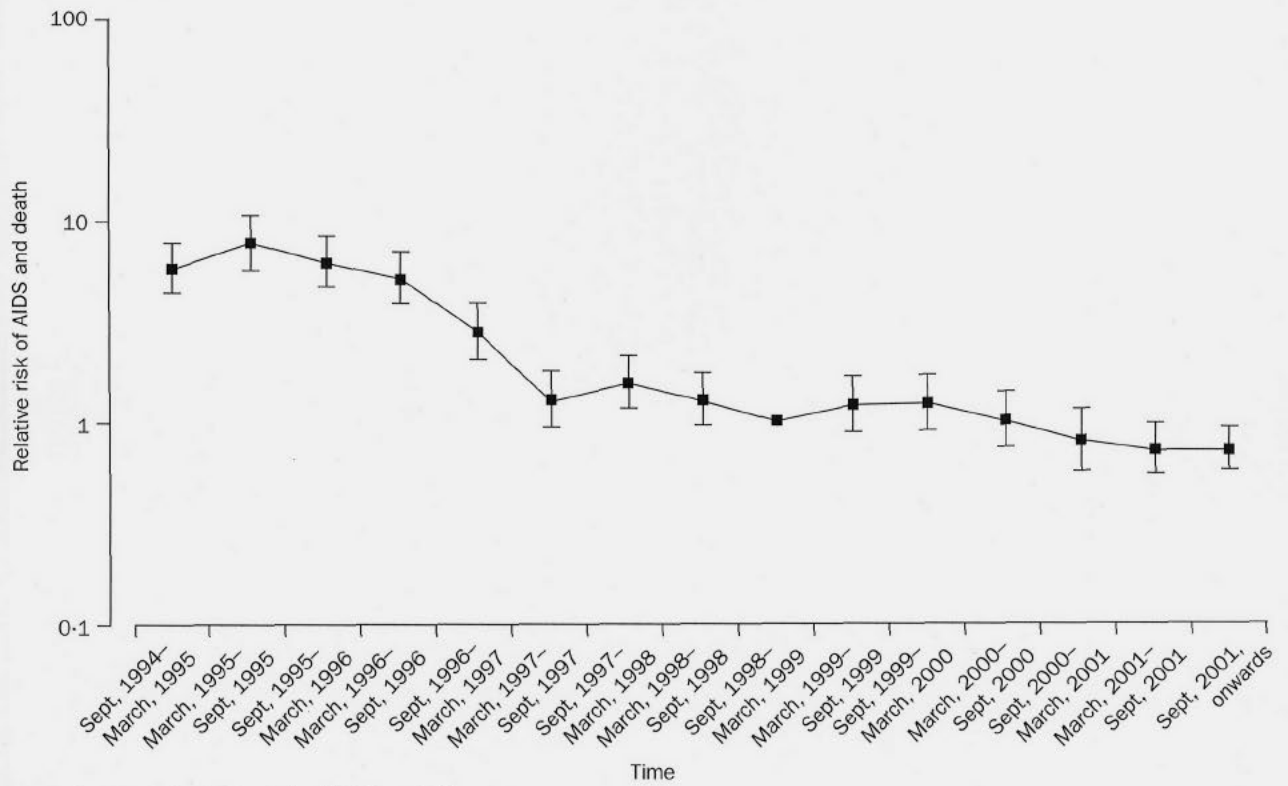

Figure 3: **Adjusted relative risk of AIDS or death**
Vertical bars=95% CIs. Data adjusted for CD4 count at recruitment, age, previous HAART treatment, AIDS status (yes or no).

early-HAART to late-HAART eras. The incidence of AIDS was roughly 50% lower in the late-HAART era than in the early-HAART era, irrespective of the latest CD4 count ($p<0·0001$).

Table 3 shows the relative risk of AIDS, all deaths, or HIV-1 related deaths separately, according to treatment era. Early-HAART was used as the reference period. In univariate analyses, the relative risk of AIDS increased during the pre-HAART period (RH 2·95, 95% CI 2·63–3·32, $p<0·0001$) and decreased in the late-HAART period (0·27; 0·24–0·31, $p<0·0001$). After adjustment for CD4 count at recruitment, age, AIDS status at recruitment, and whether the patient had started HAART at recruitment or not, there remained an increased risk of AIDS in the pre-HAART era (1·39, 1·16–1·67, $p=0·0004$) and a reduced risk of both in the late-HAART

Latest CD4	Pre-HAART (95% CI)	Early-HAART (95% CI)	Late-HAART (95% CI)	Test for trend p value*	Test for trend p value†
All deaths					
Latest CD4 (cells μL)					
≤20	68·9 (62·8–75·0)	80·0 (71·5–88·5)	34·6 (28·6–40·6)	<0·0001	<0·0001
21–50	32·2 (26·9–37·5)	28·1 (22·7–33·5)	25·7 (20·5–30·9)	0·0083	0·52
51–100	21·1 (16·7–25·5)	9·5 (7·1–11·9)	8·3 (6·3–10·3)	<0·0001	0·44
101–200	5·9 (4·2–7·6)	4·0 (3·0–5·0)	4·0 (3·3–4·7)	0·046	0·95
201–350	2·7 (1·7–3·7)	1·4 (0·9–1·5)	1·4 (1·1–1·7)	0·013	0·89
>350	1·4 (0·6–2·5)	1·2 (0·7–1·8)	0·7 (0·6–0·8)	0·008	0·041
Total	19·0 (17·7–20·3)	9·3 (8·6–10·0)	2·6 (2·4–2·8)	<0·0001	<0·0001
HIV-related deaths					
Latest CD4 (cells μL)					
≤20	53·8 (48·4–59·2)	66·8 (59·1–74·6)	26·2 (20·9–31·4)	<0·0001	<0·0001
21–50	22·9 (18·4–27·4)	21·6 (16·8–26·3)	11·9 (8·4–15·4)	<0·0001	0·002
51–100	16·0 (12·2–19·9)	7·3 (5·2–9·3)	5·5 (3·9–7·2)	<0·0001	0·19
101–200	4·7 (3·2–6·2)	2·5 (1·7–3·3)	2·2 (1·7–2·8)	<0·0001	0·54
201–350	1·8 (1·1–2·7)	1·2 (0·7–1·7)	0·8 (0·5–1·0)	<0·0001	0·048
>350	0·9 (0·4–2·0)	1·0 (0·5–1·6)	0·4 (0·3–0·5)	<0·0001	<0·0001
Total	14·6 (13·4–15·8)	7·4 (6·8–8·1)	1·5 (1·4–1·7)	<0·0001	<0·0001
AIDS					
Latest CD4 (cells μL)					
≤20	97·9 (88·6–107·2)	103·2 (91·5–114·9)	50·4 (41·5–59·3)	<0·0001	<0·0001
21–50	64·8 (56·0–73·6)	52·7 (44·2–61·2)	23·4 (18·0–28·8)	<0·0001	<0·0001
51–100	42·4 (35·5–49·3)	24·7 (20·6–28·3)	10·5 (8·1–12·9)	<0·0001	<0·0001
101–200	15·9 (13·0–18·8)	7·6 (6·2–9·0)	4·3 (3·5–5·1)	<0·0001	<0·0001
201–350	6·1 (4·6–7·6)	3·8 (2·9–4·7)	1·5 (1·2–1·8)	<0·0001	<0·0001
>350	3·6 (2·2–5·0)	2·6 (1·8–3·4)	0·7 (0·5–0·9)	<0·0001	<0·0001
Total	27·4 (25·7–29·1)	13·4 (12·5–14·3)	2·6 (2·4–2·8)	<0·0001	<0·0001

PYFU=person-years of follow-up. *Pre-HAART *vs* early-HAART *vs* late-HAART. †Early-HAART *vs* late-HAART.

Table 2: **Incidence per 100 PYFU of AIDS, all deaths, and HIV 1 related deaths according to treatment era and latest CD4 count**

	AIDS		All deaths		HIV-1-related deaths	
	RH (95% CI)	p	RH (95% CI)	p	RH (95% CI)	p
Univariate						
Pre-HAART	2·95 (2·63–3·32)	<0·0001	2·75 (2·41–3·14)	<0·0001	2·64 (2·27–3·07)	<0·0001
Early-HAART	1·00 (-)	..	1·00 (..)	..	1·00 (..)	..
Late-HAART	0·27 (0·24–0·31)	<0·0001	0·27 (0·24–0·32)	<0·0001	0·22 (0·19–0·26)	<0·0001
Multivariate*						
Pre-HAART	1·39 (1·16–1·67)	0·0004	1·29 (1·08–1·56)	0·0065	0·91 (0·72–1·13)	0·31
Early-HAART	1·00 (-)	..	1·00 (..)	..	1·00 (..)	..
Late-HAART	0·62 (0·50–0·77)	<0·0001	0·66 (0·53–0·82)	0·0002	0·76 (0·59–1·00)	0·049
Multivariate†						
Pre-HAART	1·31 (1·10–1·57)	0·0032	1·18 (0·97–1·45)	0·069	0·88 (0·70–1·09)	0·24
Early-HAART	1·00 (-)	..	1·00 (..)	..	1·00 (..)	..
Late-HAART	0·72 (0·58–0·90)	0·0035	0·84 (0·67–1·06)	0·14	1·06 (0·76–1·31)	0·98

*Adjusted for CD4 count, age, previous treatment, starting HAART, AIDS status; all at recruitment. †Adjusted as for * and additionally for latest CD4 count as a time-dependent covariate.

Table 3: **Relative hazard (RH) of AIDS or death according to treatment era**

era (0·62, 0·50–0·77, p<0·0001). Adjustment for the most recent CD4 count as a time-dependent covariate did not substantially alter the relative risk of AIDS in either the pre-HAART or late-HAART treatment era. Thus, the late-HAART treatment era had a significantly reduced risk of AIDS compared with the early-HAART treatment era.

Slightly different results were observed when looking at the relative risk of all deaths or HIV-related deaths. After adjustment for CD4 count at recruitment, age at recruitment, AIDS status at recruitment, and whether the patient had started HAART at recruitment or not, there remained an increased risk of any death in the pre-HAART era (RH 1·29, 95% CI 1·08–1·56, p=0.0065) and a reduced risk in the late-HAART era (0·66, 0·53–0·82, p=0·0002) compared with the early-HAART era. However, after adjustment for the latest CD4 count, there were no significant differences in the risk of death in either the pre-HAART era (1·18, 0·97–1·45, p=0·069) or the late-HAART era (0·84, 0·67–1·06, p=0·14) when compared with the early-HAART era. More specifically for HIV-1 related deaths, after adjustment for factors known at recruitment, there was no increased risk of HIV-1 related death in the pre-HAART era, but a reduced risk in the late-HAART treatment era, although because non-HIV-1 related deaths were excluded, the power to detect differences was reduced. Further, after adjustment for current CD4 count, there were no differences in the risk of HIV-1 related deaths in either the pre-HAART or late-HAART treatment era.

Discussion

The results of this study with over 30 000 PYFU show that the combined incidence of AIDS and death has continued to fall in the later years of HAART. Although changes in CD4 counts could account for most of the differences in relative risk of death in different treatment eras, they did not explain the differences in the relative risk of AIDS.

Since the initial fall in AIDS and death rates associated with the introduction of HAART,[1-4] few studies have a longitudinal follow-up over several years to monitor changes. Clinical event rates are now so low that most studies concentrate on virological and immunological endpoints.[14-16] The rates of AIDS and deaths were first combined to give an overall picture, and then split according to treatment era, in recognition that AIDS has become less of a distinct event in HIV-1 infection and also that death rates might change because of other, non-AIDS defining events. Decisions to start treatment might be based on factors other than CD4 count or viral load, and a population-level analysis that uses treatment eras allows comparison of event rates within a population who should have broadly the same access to therapy. Results from a study from the ART collaboration group[17] who used data for individual patients, show findings highly consistent with ours. The decline in event rate could be attributable to several factors.

Cohorts IV and V were not required to have a CD4 count of below 500 cells/μL at recruitment, which might account for some of the improvement in morbidity and mortality that we noted, because the short-term risk of a clinical event is still thought to depend mainly on the CD4 count rather than viral load.[18-20] However, the median CD4 count of cohorts I–III had increased substantially by the time of recruitment to cohorts IV and V (data not shown). This finding suggests that one reason for the increase in the population CD4 count is a general rise in CD4 counts attributable to HAART, rather than changes in the way patients were selected. Because of the increase in CD4 count over time in the early cohorts by the time of recruitment to cohorts IV and V, adjustment for the CD4 count at recruitment to these later cohorts might have resulted in underestimation of the reduction in mortality and morbidity. Despite this conservative bias, an important finding was the pronounced decline in the risk of AIDS or death during 1994–1997 and a less striking, but significantly further reduced risk of AIDS or death after September, 2000.

The continued fall in morbidity and mortality in the late-HAART treatment era could also relate to increased experience of treatment with HAART, better understanding and management of complicated drug regimens and toxicities,[10] and of the role of drug resistance.[21,22] Additionally, the availability of new drugs such as efavirenz, abacavir, and kaletra,[23] and use of HAART without previous antiretroviral treatment (ie, treatment naive), or with antiretrovirals not previously given,[8,24] might all have contributed to improved long-term adherence and subsequent survival. Importantly, problems of serious adverse events, adherence to complicated regimens, and absence of virological effect have not yet affected mortality and morbidity in the population.

When follow-up was stratified by latest CD4 count and treatment era, the incidence of AIDS was consistently lower (by about 50%) within every CD4 count strata in comparison of early and late-HAART. The median CD4 count within each strata was very similar (except for the group with a CD4 count higher than 350 cells/μL), and hence this cannot explain the lower rates in the late-HAART era. The EuroSIDA study was closely monitored, and the lower rates of AIDS diagnoses are unlikely to be due to under reporting. Nor can they be

explained by lower post-mortem rates, since diagnoses made at death were excluded. Sensitivity analyses that used different censoring strategies revealed highly consistent results (data not shown). Possibly, the spectrum of severe clinical disease associated with HIV-1 is changing. Illnesses associated with advanced HIV-1 disease during the early part of the epidemic (such as cytomegalovirus and atypical mycobacterial disease) are now rarely seen, but diseases of specific organs (kidney, liver, heart) or non-AIDS-defining diseases might arise more frequently and could be associated with substantial morbidity and mortality.[25–27] The further decrease in rates in the late-HAART period might also indicate the time needed for an immunological response; possibly, these changes show the longlasting effect of HAART rather than initial response to the drug.

The fact that the incidence of all deaths did not continue to decrease in the late-HAART era in patients with higher CD4 counts (≥20 cells/μL) might indicate mortality from causes other than specific AIDS-defining illnesses.[28] Death rates in people with severe immunodeficiency have greatly decreased in the late-HAART era and this might be due to the growing number of regimens available and increasing experience with salvage therapy.[29] This reduced rate might also indicated that HAART has a clinical benefit over and above that measured by CD4 count and viral load.[30] We noted a decrease in HIV-1 related deaths among some patients in the late-HAART compared with the early-HAART era. This reduction was not seen for patients whose CD4 count at the time of analysis was 51–20 cells/μL which might be partly due to fewer HIV-related deaths in this group compared with others. In models that adjusted for factors known at recruitment, there was a reduced risk of HIV-1 related death in the late-HAART era compared with the early-HAART era, suggesting a real decrease in these types of deaths that cannot be explained by differences in treatment or values of immunosuppression at recruitment.

Several important points should be considered in interpretation of our results. Is the EuroSIDA population in any one 6-month period representative of that in European clinics in general? Although many centres across Europe are represented in this study, centres that were not included might differ in terms of experience of clinician or availability of HAART regimens. Treatment decisions are specific to the treatment centre and might not be uniform across Europe. Additionally, our cohort differs slightly from clinic populations in that we have five distinct cohorts of patient entry, compared with an open clinic in which new patients are enrolled continuously. However, the selection of consecutive patients should mean that our cohort is broadly representative of those who are regularly seen, and, because of the large number of clinics in the study, arguably more representative than any one clinic cohort.

Nonetheless, the AIDS and death rates in EuroSIDA are very similar to those in other, open cohorts.[3,4,31] Loss to follow-up can affect the results of cohort studies, but, in EuroSIDA there was a low loss-to follow-up rate (defined as a patient not known to have died and with no follow-up after January, 2001) of about 10%. Loss to follow-up was higher among intravenous drug users than in all other exposure groups (rates of 14% and 9%, respectively). Further information on patients with no follow-up visit for over 12 months was sought in a consistent and uniform way. However, the length of the study allowed us to monitor long-term trends and changes in morbidity and mortality, and regular data monitoring ensures the ongoing accuracy of the data.

Adjustment for viral load in temporal analyses such as these is seriously limited due to the strong association between calendar time and the introduction of viral load testing. In general, we have not adjusted our results for viral load but when we did adjust for viral load, we recorded similar results. However, the number of patients included in the analysis was reduced by over 50%, the events were reduced by an even greater proportion (because most of the events occurred in the time before routine viral load testing), and the analysis excluded most people recruited before 1997. As follow-up and events accumulate in the late-HAART era, further work could look at the relationship between disease progression, calendar time, and markers of disease progression.

In conclusion, the combined incidence of AIDS and death has declined in patients in the EuroSIDA study. Although the incidence of AIDS declined further in the late-HAART era, we noted little decline for all deaths, and some decline in HIV-1 related deaths, especially among patients with low CD4 counts. The introduction and continued use of HAART over the past 6 years has resulted in very low morbidity and mortality rates across Europe, suggesting that limitations of current treatment, including potential adverse effects of long-term HAART and problems with compliance, have not yet affected the clinical success of HAART in the population. Long-term follow-up of large cohorts such as those in EuroSIDA should prove essential to observe changing causes or morbidity and mortality among patients with HIV.

Contributors

A Mocroft proposed the topic, did statistical analyses, and was mainly responsible for writing the report. C Katlama, P Reiss, A d'Arminio Monforte, B Knysz, and M Dietrich contributed ideas for analyses; provided input into writing the manuscript; and contributed to protocol design, national or local study design, and data collection. A Phillips supervised statistical analyses, contributed to study design and writing the report. B Ledergerber and O Kirk contributed ideas for study design and analyses, writing of the manuscript, and overall study design and co-ordination. J Lundgren, as overall study coordinator, contributed to study design, statistical analyses, drafting the manuscript, and supervised the project.

Conflict of interest statement

B Ledergerber received travel grants from Abbott, Roche, GlaxoSmithKline, BMS, and MSD. A Phillips has received research grants, consultancy fees, or speaker honoraria from Roche, GlaxoSmithKline, Boehringer Ingelheim, Abbott, and BMS. None of the other authors has declared any conflict of interest.

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¿Por qué se infectan aún niños con el virus de la inmunodeficiencia humana en España?

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Introducción

A pesar del éxito en la prevención de la transmisión vertical (TV) del virus de la inmunodeficiencia humana tipos 1 y 2 (VIH-1 y 2) en los países desarrollados, todavía siguen naciendo niños infectados. El propósito de este análisis es evaluar los fallos en la prevención de la TV y las características de los niños infectados

Métodos

La Cohorte FIPSE de Madrid sigue prospectivamente a los niños hijos de gestantes VIH que nacen en 8 hospitales públicos de Madrid. Desde mayo de 2000 hasta diciembre de 2005, se siguieron 632 niños. Se han analizado los datos de la gestación, seguimiento, tratamiento antirretroviral (TAR), y clínica al diagnóstico de los casos de TV.

Resultados

Se infectaron 9 niños. La tasa de TV fue del 1,42 % (intervalo de confianza [IC] 95 %: 0,7-2,68). 7/9 madres no recibieron TAR durante la gestación (y de ellas, cinco tampoco lo recibieron en el parto). De las madres que recibieron TAR, una sólo cumplió un mes de tratamiento. Dos niños recibieron triple terapia como prevención de la TV, un niño recibió biterapia y, el resto, monoterapia. La mediana de edad al diagnóstico fue de 2,4 meses (rango: 7 días-2 años). La carga viral media en el momento del diagnóstico fue de 276.000 copias/ml (rango: 11.900-1.000.000). Un total de 5/9 de los casos eran sintomáticos al diagnóstico (2 neumonías por *Pneumocystis jiroveci,* una sepsis, una infección bacteriana de repetición, una hepatoesplenomegalia). Un total de 4/9 requirieron ingreso hospitalario antes del diagnóstico de VIH.

Discusión

Se identificaron "oportunidades perdidas" de prevención de la TV en 8 de los 9 niños infectados (89 %). El uso de zidovudina durante el parto y la triple terapia al recién nacido de riesgo no están universalmente extendidos. El ingreso hospitalario de lactantes en riesgo de TV debería hacer sospechar una posible infección. Se debería reforzar el acceso y la implementación de todas las medidas de prevención de la TV en el sistema sanitario.

Palabras clave:

VIH. Transmisión vertical. Tratamiento antirretroviral.

WHY ARE HIV-INFECTED INFANTS STILL BEING BORN IN SPAIN?

Introduction

Despite the success of preventive measures against mother-to-child transmission (MTCT) of human immunodeficiency virus-1 and -2 (HIV-1 and -2) in developed countries, HIV-infected infants continue to be born. The aim of this study was to evaluate failures in the prevention of MTCT and the clinical characteristics of infected infants.

Methods

The Foundation for the Investigation and Prevention of AIDS in Spain (FIPSE) Cohort in Madrid prospectively follows up children at risk of MTCT HIV born in eight public hospitals in Madrid. From May 2000 to December 2005, 632 children born to HIV-infected mothers were evaluated. Data from pregnancy follow-up, antiretroviral therapy

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(ART), and symptoms at diagnosis in infected infants were analyzed.

Results

Nine infants were infected. The rate of vertical transmission was 1.42 (95% CI 0.7-2.68). Of the nine mothers, seven had not received ART during pregnancy (and five had not received ART at delivery). Of the mothers who received ART, one had only done so for the last month of pregnancy. Two infants were given three drugs as prevention of MTCT, one received bitherapy and six received monotherapy. The median age at diagnosis was 2.4 months (range 7 days-2 years). The mean plasma viral load at diagnosis was 276,000 copies/ml (range: 11,900-1,000,000). Five of the infants were symptomatic at diagnosis (*P. jirovaci* pneumonia in two, sepsis in one, recurrent bacterial infections in one, hepatosplenomegaly in one). Four of the nine infants had been admitted to hospital prior to HIV diagnosis

Discussion

Missed opportunities for the prevention of MTCT were identified in eight of the nine HIV-infected infants (89%). Administration of AZT during labor in HIV-infected mothers and triple therapy for the prevention of MTCT in high risk infants is not universal. Hospital admission in young infants at risk might lead to suspicion of infection in infants born to HIV-infected mothers. Improved implementation of all the preventive measures for MTCT should be encouraged.

Key words:

HIV. Mother-to-child transmission. Antiretroviral treatment.

INTRODUCCIÓN

La infección en niños por el virus de la inmunodeficiencia humana (VIH) tipo 1 es debido casi exclusivamente a la transmisión vertical (TV) de gestantes VIH positivas a sus hijos[1]. La epidemiología de la TV del VIH ha cambiado radicalmente en los últimos 10 años. Antes del desarrollo de intervenciones específicas para prevenir la TV, un 15-25% de los niños en Europa y Estados Unidos y un 30-40% de los niños nacidos en África subsahariana se infectaban[2]. Desde la implementación a partir de 1994 del régimen PACG076, que incluye la terapia con zidovudina (AZT) en la gestación, la administración de AZT en el parto y al niño durante 6 semanas y la lactancia artificial, la TV se redujo al 6,7%. Al añadir la cesárea a este protocolo, la tasa disminuyó al 2%, y con la administración de tratamiento antirretroviral de gran actividad (TARGA) durante la gestación, esta tasa se sitúa cercana al 1%[1-8]. En los últimos años se han sucedido distintas guías de actuación clínica en la prevención de la TV, tanto en países en vías de desarrollo como en países desarrollados. La Guía Británica del año 2005 aconseja actualmente el cribado del virus en toda gestación y el TARGA de todas las gestantes VIH positivas[6]. Así mismo, permite el parto por vía vaginal si la carga viral (CV) es indetectable en el tercer trimestre; y en este caso, aconseja tratamiento al recién nacido con monoterapia con AZT durante 4-6 semanas. Entre las medidas de prevención de la TV, siempre según esta guía, también se incluye triple terapia (con AZT, nevirapina [NVP] y lamivudina [3TC]) en los recién nacidos de alto riesgo (cuando la madre no ha recibido tratamiento durante la gestación, la CV plasmática no es indetectable en el tercer trimestre o la infección materna se ha diagnosticado tras el parto[6]). Esta última medida preventiva (el tratamiento con 3 fármacos al recién nacido de alto riesgo) no se contempla, sin embargo, en el último informe sobre la prevención de la TV elaborado en el año 2004 por la American Academy of Pediatrics, donde se aconseja exclusivamente el uso de monoterapia o biterapia para este grupo de recién nacidos[7]. Así mismo, las recomendaciones del Public Health Service Task Force de Estados Unidos, en su última revisión de julio de 2006, tampoco contemplan la triple terapia en el recién nacido de riesgo y siguen proponiendo el uso exclusivo de AZT durante 6 semanas para los recién nacidos de gestantes que no han recibido tratamiento antirretroviral (TAR) durante la gestación, e incluso para recién nacidos de gestantes que no lo recibieron tampoco en el parto[8]. Esta guía propone a lo sumo un tratamiento corto (de una semana) con biterapia (AZT y 3TC) en recién nacidos de madres que no recibieron tratamiento en la gestación pero sí en el parto.

A pesar de la implementación de estas medidas, siguiendo unas u otras guías, en los servicios de salud españoles, aún hay casos de TV. El objetivo de este trabajo es analizar dichos casos en una cohorte de niños nacidos de madre VIH positiva, evaluar la carga que supone cada factor u "oportunidad perdida" en la prevención de la TV y describir las características clínicas iniciales de los recién nacidos infectados.

MÉTODOS

Se seleccionaron los 9 casos de TV entre los 632 niños con exposición perinatal a VIH recogidos desde mayo de 2000 hasta diciembre de 2005 en la base de datos de las Cohorte FIPSE (Fundación para la Investigación y la Prevención del SIDA en España) del Proyecto 36299/02 (Seguridad del Tratamiento Antirretroviral en Madres Gestantes VIH y Transmisión Vertical) en el que colaboran diversos hospitales de la Comunidad Autónoma de Madrid (Hospital 12 de Octubre, Hospital La Paz, Hospital Gregorio Marañón, Hospital Universitario de Getafe, Hospital Severo Ochoa, Hospital de Móstoles, Hospital Príncipe de Asturias y Hospital Fundación Alcorcón. Las características de las gestantes seguidas en dicha cohorte han sido publicadas recientemente[9], incluyéndose en ella a las gestantes VIH positivas identificadas antes de la gestación, durante la misma o en las 48 h posteriores al parto. Los pares de madres-niños son seguidos prospectivamente, aunque en esta serie se han analizado los casos de TV

de manera retrospectiva. En el seguimiento de las madres se recogen datos sobre el control de la gestación, tratamiento antirretroviral recibido, las analíticas correspondientes a cada visita programada, los efectos adversos observados y las características del parto. En el seguimiento de los niños se anotan las características antropométricas, el tratamiento recibido desde el nacimiento, y los datos clínicos y analíticos correspondientes a cada visita, que se efectúa al nacimiento, a las 2-3 semanas, a las 6 semanas, a los 3, 6, 12, 18, 24 y 36 meses. La analítica incluye reacción en cadena de la polimerasa (PCR) para VIH en todas las visitas hasta la de los 3 meses, inclusive. Hasta el año 2004 se realizaba PCR del ADN proviral (del virus integrado en las células mononucleadas) casi exclusivamente, como método de cribado, cualitativo. En la actualidad, este método ya no está disponible y se utiliza como método de cribado la PCR del ARN viral, que cuantifica la CV plasmática. Desde el nacimiento, se suspende la lactancia materna; se descarta de manera definitiva la infección por VIH al producirse la serorreversión entre los 18 y los 24 meses.

Para el cálculo de los intervalos de confianza se usó el programa estadístico GraphPad, disponible *online* en www.graphpad.com/quickcalcs

Resultados

En el período estudiado (mayo 2000-diciembre 2005), en la Comunidad Autónoma de Madrid nacieron 17 niños infectados por VIH. De ellos, nueve pertenecen a la Cohorte FIPSE. El resto o bien pertenecen a otros hospitales o bien no cumplen los criterios de inclusión en la Cohorte (madres identificadas varios meses tras el parto, por fallo en la detección durante la gestación).

La tasa neta de Transmisión Vertical de VIH en la Cohorte y en el período de estudio fue de 1,42% (IC 95%: 0,7-2,68) (9 casos de TV entre un total de 632 pares madre-niño).

De las 9 gestaciones, únicamente tres fueron controladas. El resto de las gestantes, no acudió a los controles de la gestación previstos ni solicitó ayuda de los servicios sanitarios, debido a problemas de índole social como drogadicción (casos 1, 2, 6 y 8), marginalidad (caso 8), ocultamiento del embarazo (caso 7) e inmigración ilegal (caso 5). Entre las madres que sí controlaron su gestación, dos recibieron TAR (en ambas ocasiones consistió en triple terapia, incluyendo un inhibidor de la proteasa [IP]). Y sólo una de estas 2 madres tomó TARGA durante toda la gestación (tabla 1). Esta gestante (caso 9) tomaba inicialmente atazanavir, un IP que fue sustituido por nelfinavir al diagnosticarse el embarazo. Este último fue mal tolerado y se pautó de nuevo atazanavir, que tomó durante los últimos 4 meses de la gestación junto con otros 2 análogos de nucleósidos. Las CV de esta gestante fueron en todo momento negativas.

Sólo una de las 9 gestantes era inmigrante de raza negra (procedía de Camerún, caso 5). En el resto de los casos, se trataba de mujeres españolas. El diagnóstico materno de infección VIH se realizó tras el parto en 3 de los 9 casos (en las primeras 48 h de vida del recién nacido). El 44% de las gestantes estaba coinfectado con virus de la hepatitis C (VHC). En 5 casos el parto fue por vía vaginal (en uno de ellos [caso 2] fue debido a la escasez de tiempo para realizar cesárea al ingresar la madre en expulsivo; en tres de ellos por desconocimiento de la seropositividad materna hasta el posparto y en uno de ellos por presentar CV indetectable). No se administró AZT intravenosa durante el parto a 5 de los casos (bien por falta de tiempo [caso 8], bien por desconocimiento de la infección materna [casos 5, 6, 7] o bien por ocultación de la seropositividad [caso 1]) (tabla 1). Dos de los recién nacidos (casos 1 y 6) fueron prematuros, con edades gestacionales de 28 y 32 semanas y con un peso medio de 1.112 g (tabla 2).

Respecto al TAR en el recién nacido, 2 recién nacidos recibieron triple terapia con AZT, nevirapina y lamivudina por ser de alto riesgo (no habían recibido TAR durante la gestación ni durante el parto). Un niño recibió biterapia (con AZT y lamivudina) y el resto monoterapia con AZT (tabla 2).

TABLA 1. **Características de la gestación, parto y tratamiento del recién nacido**

Caso	Diagnóstico madre	VHC madre	Control gestación	TAR materno	AZT parto	Cesárea	Bolsa rota (h)	TAR niño
1	Pregestación	No	No	No	No	Sí	> 72	Bi
2	Pregestación	Sí	No	No	Sí	No	0	Mono
3	Pregestación	No	Sí	Sí *	Sí	Sí	0	Mono
4	Pregestación	Sí	Sí	No	Sí	Sí	0	Mono
5	Posparto	No	No	No	No	No	¿?	Mono
6	Posparto	No	No	No	No	No	4	Mono**
7	Posparto	No	No	No	No	No	2	Triple
8	Pregestación	Sí	No	No	No	Sí	4	Triple
9	Pregestación	Sí	Sí	Sí	Sí	No	¿?	Mono

*Mala adherencia.
**Inicio de TAR a los 6 días de vida.
VHC: virus de la hepatitis C; TAR: tratamiento antirretroviral; AZT: zidovudina.

TABLA 2. **Características clínicas y analíticas de los casos**

Caso	Peso RN (g)	Edad diagnóstico (meses)	CD4 diagnóstico	CV ($\times 10^3$)	PCR VIH necesarias	Clínica	Ingreso UVI	Analítica
1	1.049	2,6	46%	1.000	4	Sepsis	Sí	Ø
2	2.735	2,4	31%	336	3	ITU, GEA por *C. jejuni*, OMA	No	Hb: 8,1
3	3.240	0,2	54%	179	1	Ø	No	Ø
4	2.950	6,2	28%	97	3	NPj*	Sí	Hb: 7,9; GGT 580
5	2.930	24,1	12%	243	5	Ø	No	GOT 88; GPT 81
6	1.174	1,2	42%	100	2	Hígado ↑ Bazo ↑	No	Ø
7	3.190	0,3	35%	25	2	Ø	No	Ø
8	2.440	0,3	36%	11,9	1	Ø	No	Ø
9	3.650	2	?	?	2	NPj → †**	No	?

*Neumonía por *P. jiroveci*.
**Fallece por neumonía por *P jiroveci*.
Ø: Sin hallazgos significativos; ?: Sin datos.
RN: recién nacido; Hb: hemoglobina; CV: carga viral; PCR: reacción en cadena de la polimerasa; VIH: virus de la inmunodeficiencia humana; ITU: infección del tracto urinario; GEA: gastroenteritis aguda; OMA: otitis media aguda; UVI: unidad de vigilancia intensiva.

La mediana de edad al diagnóstico fue de 2,4 meses (rango: 7 días-21 meses). Una niña fue diagnosticada a los 2 años de vida a pesar de tener 4 PCR-ADN de VIH negativas realizadas en los primeros 4 meses posparto. En este caso, la permanencia de los anticuerpos anti-VIH después de los 2 años llevó a la petición de ARN-PCR de VIH (cuantitativo, más sensible que el anterior) que fue positivo para VIH-1, subtipo A/G recombinante.

Respecto al momento de la transmisión, podemos suponer que en un tercio de los casos (3, 7, 8), la transmisión habría sido intraútero, ya que el virus es detectable en la primera semana de vida

Las muestras de PCR de VIH necesarias para llegar al diagnóstico fueron 2,5 como media (rango: 1-5) y la CV inicial osciló entre 11.900 y 1.000.000 copias/ml (media: 276.000) (tabla 2).

El porcentaje de linfocitos T CD4+ medio de los niños al diagnóstico fue de 38% (rango: 12-54%).

Cinco de los casos eran ya sintomáticos cuando se realizó el diagnóstico de infección VIH. Dos de ellos (casos 4 y 9), presentaron neumonía por *Pneumocystis jiroveci*. Uno presentó sepsis clínica, uno infecciones bacterianas de repetición y uno hepatoesplenomegalia. De ellos, 4 niños habían precisado ingreso hospitalario por causa infecciosa (síndromes febriles sin foco o infección respiratoria grave) antes del diagnóstico de infección por VIH. El caso 9, inicialmente asintomático, fue llevado a la urgencia hospitalaria con insuficiencia respiratoria grave y parada cardiorrespiratoria posterior, que no pudo revertirse, falleciendo instantes después, a la edad de 4 semanas. La necropsia identificaría neumonía por *P. jiroveci*. En la evolución posterior de los otros 8 niños, no ha habido ningún otro fallecimiento.

Respecto a los hallazgos analíticos, en 4 casos se apreciaron alteraciones hematológicas (anemia moderada en los casos 2 y 4, con cifras de hemoglobina de 8,1 y 7,9 mg/dl, respectivamente) o bioquímicas (hipertransaminasemia en los casos 4 y 5) (tabla 2).

Discusión

La tasa de TV en la cohorte FIPSE de Madrid es similar a la de otras cohortes europeas[10-13] donde se recoge el seguimiento de gestantes VIH positivas y de sus recién nacidos. Esta tasa (1,42%) no refleja la efectividad de las medidas de prevención de la TV, ya que los casos de TV son el resultado, en su gran mayoría (8 de 9) de "oportunidades perdidas" en la prevención: gestaciones mal controladas (o con mala adherencia al TAR).

A la vista de los datos y en el período estudiado, se debe destacar como factor causal más importante la falta de TAR durante la gestación. Sólo 1 de las 7 gestantes que no tomaron TAR era inmigrante. El resto eran mujeres españolas, potenciales usuarias del sistema de salud en nuestro país, y que por diversos motivos (ocultación del embarazo, drogadicción, marginalidad) no solicitaron ayuda en el seguimiento de su gestación.

Un alto porcentaje de las madres (44%) estaba coinfectado por VHC.

Es de destacar también que en esta serie de casos de TV, ninguno corresponde a niños de gestantes diagnosticadas durante la gestación. El estudio colaborativo europeo, sin embargo, encuentra una tasa mayor de TV en las gestantes que iniciaron TARGA durante la gestación (1,92%) que en aquellas que lo iniciaron previamente (0,25%)[12].

La CV indetectable es el objetivo del tratamiento en la mujer gestante VIH. Aunque la TV puede ocurrir incluso cuando la CV es indetectable[5,6,14-16], la tasa de TV aumenta 2,4 veces por cada aumento de un logaritmo en la CV plasmática[14]. Según las recomendaciones de la British HIV Association[6] y las del Public Health Service Task Force de Estados Unidos[8], cuando una gestante VIH presen-

ta una CV indetectable en el último trimestre, el parto por vía vaginal ha de ser la norma, salvo deseo contrario de la madre. Sin embargo, en el último informe del Grupo Colaborativo Europeo, la cesárea programada se asoció a una disminución del 90 % en las pacientes con CV indetectable[12]. Estos resultados aún tienen que contrastarse y es aún muy prematuro indicar cesárea sistemática en todas las gestantes VIH positivas independientemente de su CV, aunque podría ser la norma en el futuro.

Una de las gestantes de nuestra serie (caso 9) transmitió la infección a su recién nacida, a pesar de contar con una CV plasmática indetectable (< 50 copias/ml) tras recibir TARGA durante la gestación. Además, se administró AZT intravenosa intraparto y oral al recién nacido durante 6 semanas. La vía elegida para el parto fue la vaginal, siguiendo las recomendaciones actuales para dichas gestantes[6]. Sin embargo, en casos aislados, la CV plasmática podría no representar la actividad del virus en el tracto genital femenino (TGF) y eso podría justificar los raros casos de transmisión vertical en gestantes con CV plasmática indetectable (se ha establecido una tasa de TV en torno al 0,6 % en gestantes con CV < 1.000 que reciben TARGA)[6,16,17]. Aunque las cifras de CV plasmática y la existencia de virus (bien en la fracción celular como provirus o en la fracción libre de células) en las secreciones cervicovaginales están correlacionadas[18-20], una CV plasmática indetectable no garantiza ausencia del virus en el TGF: hasta en el 30 % de las mujeres con CV indetectable se encontró secreción de virus en las secreciones cervicovaginales[20]. Por lo tanto, el TAR y la administración de AZT intraparto no garantizaría la ausencia de VIH en el TGF.

En 5 casos de nuestra serie, no se llevó a cabo la administración de AZT intravenosa intraparto. Como se describe en el estudio descriptivo de las madres de la cohorte FIPSE[9] un 6 % de las madres no recibieron tratamiento en el parto, bien por falta de tiempo al presentarse la gestante ya en período expulsivo o por ser la seropositividad para VIH desconocida. Se desconoce cuál es el grado de prevención de esta medida, aunque pudiera ser el factor menos influyente en la prevención de la TV: en las guías que se han mencionado previamente, y basándose en datos de la Cohorte Perinatal Francesa[21] la administración de AZT intravenosa intraparto se considera "no esencial" en el grupo de mujeres con TARGA y CV < 50 copas/ml[6].

No se ha definido aún el papel del TAR preventivo en el recién nacido en la reducción de la TV. Como se ha descrito en la introducción, existen discrepancias respecto al TAR profiláctico en recién nacidos de alto riesgo entre las recomendaciones americanas[8] y las europeas[6]. En las primeras, se baraja la biterapia (con AZT más 3TC o NVP) e incluso la monoterapia con AZT exclusivamente, en aquellos recién nacidos con alto riesgo de TV[5,7,8]. En los casos 1 al 6 (que recibieron exclusivamente monoterapia con AZT) se siguieron posiblemente estas recomendaciones americanas, más conservadoras. Los recién nacidos 7 y 8, fueron tratados con triple terapia, según las recomendaciones británicas.

En nuestra pequeña serie, la proporción de TV intraútero (un tercio de los casos) resulta algo mayor de lo esperado, ya que ésta supone habitualmente un 15-20 % de los casos de TV[2]. Estas diferencias entre nuestros resultados y lo publicado en la literatura especializada se deben probablemente al escaso número de pacientes de nuestra serie. Podemos asumir en el resto de los casos que la infección ha sido intraparto, ya que se evitó la lactancia materna en todos los recién nacidos.

El diagnóstico de infección por VIH en el caso 5 demoró 21 meses: se trataba de una niña de raza negra infectada por un subtipo viral poco común, el VIH-1 A/G. Este subtipo de virus VIH-1 grupo M "no B", pertenece a las llamadas "formas recombinantes circulantes", que recientemente han sido descritas como el subtipo de VIH-1 más predominante en países de África subsahariana como Camerún y Senegal[22]. En el estudio europeo pediátrico PENTA-5, la morbimortalidad de esta forma recombinante no fue distinta al resto de los subtipos, pero sí se observaron fallos en la detección tanto del genoma viral al diagnóstico como de resistencias frente a antirretrovirales[23]. De igual modo, se han descrito frecuentes resultados falsamente negativos en las pruebas diagnósticas de cribado en los pacientes, en su mayoría subsaharianos, infectados por subtipos de VIH-1 grupo M "no B", ya que los métodos de detección están basados en secuencias de subtipos B[24,25]. La niña, hija de madre camerunesa, presentó PCR de ADN proviral (Versant HIV-1 RNA 3.0 Assay Bayer®) negativo a los 2 y 4 meses. En este caso, el diagnóstico de infección por VIH sólo se sospechó cuando la niña mostró IgG anti VIH persistentemente elevados a la edad de 21 meses. Se realizó entonces un ARN-PCR (Amplicor HIV-1 Monitor Test 1.5 Roche®, cuantitativo, y más sensible) que mostró entonces una CV de 5,3 log copias/ml.

Las CV iniciales de todos los casos eran elevadas, como suele ser la norma[26-30]. Sorprende sin embargo que sólo 2 o 4 semanas después de obtener una primera o segunda PCR negativa, un recién nacido infectado presente CV tan elevadas: la terapia antirretroviral en el recién nacido podría negativizar la CV, retrasando el diagnóstico de infección como sugieren las conclusiones de la Cohorte Perinatal Francesa[6,31].

Es de destacar que el diagnóstico de infección por VIH se realizó durante un ingreso hospitalario por causa infecciosa en 4 de los casos (1, 2, 4, 9). Estos 4 recién nacidos empezaron en estadio clínico C antes de los 6 meses de vida. Según los resultados de las distintas cohortes de lactantes infectados por TV (en los años anteriores a la instauración del TARGA y las terapias de prevención de la TV), entre un 18 y un 25 % de ellos cumplen criterios diagnósticos de sida antes de cumplir el año de edad[26-29]. Aunque estos datos no son directamente comparables

por tratarse en nuestro caso de lactantes a los que se aplicaron diversas medidas de prevención de la TV, cabe decir que en nuestra serie, esta proporción resultó algo mayor (44%).

Respecto a las analíticas realizadas a estos niños, bien durante algún ingreso o en el seguimiento rutinario, sólo 3 casos (2, 4, 5) mostraban alteraciones como anemia o hipertransaminasemia, que, junto con la trombocitopenia y la leucopenia, son características habituales, pero no específicas, al inicio de la infección por VIH[26-31].

Este estudio está limitado por consistir exclusivamente en el análisis de una serie de 9 casos. Las conclusiones de este corte transversal pudieran no ser aplicables en otras poblaciones donde se consideraran exclusivamente objeto de estudio gestantes con TAR, o en las que se hubieran excluido a las gestantes diagnosticadas tras el parto.

En conclusión, en nuestra serie hemos identificado fallos en la prevención de la TV en el 89% de los niños infectados. El factor causal más determinante de la TV en este estudio ha sido la ausencia de TAR en las madres gestantes. Por otro lado se observa cómo el tratamiento con AZT intravenosa en el parto no alcanza a todas las gestantes seropositivas. Queremos destacar también la puesta en práctica de TAR preventivos en los recién nacidos de alto riesgo con 3 fármacos desde 2004, aunque la eficacia de esta medida no está claramente definida. También queremos llamar la atención sobre el número de PCR que fueron necesarias para llegar al diagnóstico (media: 2,5). En la práctica clínica a menudo se afirma que una segunda PCR negativa a las 6-8 semanas permite prácticamente descartar el diagnóstico. En esta serie vemos que no ha sido así. Respecto al diagnóstico, hemos observado que el ingreso por causa infecciosa de un lactante menor de 3 meses en riesgo de TV pudiera ser un factor de sospecha (datos propios no publicados).

Nuestro análisis refleja las deficiencias de la profilaxis, u "oportunidades perdidas" en una sociedad occidental con acceso libre y gratuito a todas las medidas de prevención de la TV conocidas actualmente. Son necesarias medidas que mejoren el acceso al sistema de salud y al TAR de gestantes con mal control de su embarazo, ya que son sus recién nacidos los de mayor riesgo de infección en nuestro país.

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Zidovudine Use in Pregnancy: A Report on 104 Cases and the Occurrence of Birth Defects

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Summary: As more women of childbearing age are being identified as HIV infected, vertical transmission to the fetus and/or neonate is an increasingly significant therapeutic problem. Currently the use of zidovudine is one of the few specific measures available, and as a potentially teratogenic and fetotoxic agent, any decision for its use requires evaluation of the potential for fetal damage. In a series of 104 cases of intentional or inadvertent use of zidovudine at differing gestations in pregnancy, there were eight spontaneous first trimester abortions, eight therapeutic terminations, and eight cases of fetal abnormality occurring among a total of 88 cases where the pregnancy progressed. Analysis and correlation of antenatal data and drug therapy with individual cases failed to show any specific abnormality that could reasonably be attributed to zidovudine therapy. While not proving safety, these data add to previous smaller series with similar findings, thus lending tenuous support to the use of this agent. Continuing studies are required, particularly to clarify the possibility of long-term developmental defects. **Key Words:** Pregnancy—HIV infection—Zidovudine use—Birth defects—Vertical transmission.

Currently available information concerning the use of zidovudine therapy during pregnancy and its effect on the mother and fetus/neonate is both limited and confusing. During the concentration on putative high-risk groups and practices thought to be associated with a high risk of transmission of the human immunodeficiency virus 1 (HIV-1) virus (including intravenous drug abuse and specific male homosexual practices), pregnancy and HIV-1 infection is an area that received little initial attention.

Experience worldwide has increasingly confirmed heterosexually acquired infection with vertical transmission from mother to fetus and/or mother to neonate as a major source of continuing HIV-1 infection. While raising multiple issues of importance to individuals, health authorities, and ultimately governments on the local and national levels, this also provides an opportunity for an increased understanding of the natural history of the disease together with an area of potential therapeutic intervention. In particular, intentional or inadvertent use of zidovudine in pregnancy is currently one of the few specific anti-HIV-1 measures available (1). This drug is known to pass the placenta and enter the fetal circulation, rapidly achieving levels comparable to the maternal blood level (2), so that the question of possible teratogenic and other fetal effects must be raised.

This report describes the cumulative experience from February 1990 to March 1993 in an ongoing study of pregnancy outcome in HIV-1-infected women where zidovudine has been used. The main objectives were (a) to detect any major teratogenic

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effect of zidovudine when administered inadvertently or intentionally to pregnant women infected with HIV and (b) to analyze the cumulative data on birth defects to detect any possible trend in number or type of defect and provide the basis for specific directed study, if indicated.

As the total number of pregnancies exposed to the virus and/or the drug is unknown and potential confounding factors exist, calculation of relative risk in the current context has no meaning. Nevertheless, it is important to report such outcomes as this documentation is an essential step in detecting any deviation from the background rate of fetal abnormality. Conversely, the absence of any such deviation will lend further credibility to the safety of zidovudine use in pregnancy.

METHODS

The subject population comprised a group of 104 HIV-1-infected pregnant Indian women (84 from the state of Uttar Pradesh, the remainder from Maharashtra state), who had been exposed to zidovudine at some stage of their pregnancy and were attending a sexually transmitted disease (STD) clinic at the Sanjay Ghandi Memorial Hospital in Uttar Pradesh. This is a major referral center for both states.

Forty-nine women were being treated for symptomatic HIV-1 infection [Centers for Disease Control (CDC) classification II/III], 18 for fully developed AIDS (CDC IV), 26 for asymptomatic HIV-1 infection, and five for needle stick exposure. Indication for drug therapy could not be determined in the remaining five cases. Although one case was lost to follow-up postnatally, antenatal and delivery data are known and were therefore included in the data set.

As identified cases of HIV-1 infection, specific detailed records were kept. For each case, data were abstracted to form a data sub-set for a retrospective analysis of the details regarding individual illness and drug therapy. Information collected included mother's age, symptoms related to HIV infection in the mother, zidovudine dosages, gestational age of the fetus during zidovudine treatment, any known fetal exposure to other drugs including those used to treat opportunistic infections, and substance abuse (e.g., cigarettes, alcohol, illicit drugs). This was collated with details of pregnancy outcome and evaluation of the newborn.

For the purpose of data reporting, pregnancy outcomes were categorized as one of the following: (a) outcome with birth defects, (b) outcome without birth defects, (c) spontaneous fetal loss, or (d) induced abortion.

Birth defects were defined as "any live or stillborn infant with a structural, chromosomal or biochemical abnormality diagnosed before the infant is one year of age," per the CDC Guidelines for birth defect surveillance programs (3). These guidelines also disqualify as defects those findings present in infants <2,500 g, attributable to prematurity itself, and specify an exclusion list of minor abnormalities not to be considered as birth defects. This protocol was adhered to and supervised by one of the authors (A.K.).

The outcome data are presented by the earliest trimester of zidovudine exposure. For this study, gestational age was calculated from the date of the last menstrual period, the second trimester was taken as beginning at week 13 and the third trimester as beginning at week 26. Basic criteria used for review of data were: (a) timing of exposure to zidovudine and (b) possibility of another known or likely cause (e.g., recognized genetic or chromosomal defect or exposure to a known teratogen).

RESULTS

Among the total of 104 cases, there were a number of therapeutic first trimester terminations carried out, together with an expected incidence of spontaneous abortion. As all cases were exposed to zidovudine, an initial categorization of pregnancy outcome into "with" or "without" recognized birth defect, subdivided by earliest trimester of drug exposure, is presented in Table 1. A total of eight babies affected with birth defects were recognized, their exposure to zidovudine being at different trimesters as indicated in Tables 2–4. Brief case details follow.

First Trimester

Cases A and B (Table 2)

Both mothers were treated with zidovudine in conception cycle but prior to recognition of pregnancy. The two male infants, born at full term, displayed minor anomalies. These included low-set ears, retrognathia, prominent epicanthal folds, hir-

TABLE 1. *Pregnancy outcome by earliest trimester of exposure to zidovudine*

Earliest trimester of exposure	Outcomes with birth defects (patient)	Outcomes without birth defects	Fetal wastage (status unknown)		n
			Spontaneous fetal losses	Induced abortions	
First	4 (A–D)	45	8	8	65
Second	1 (E)	5	—	—	6
Third	3 (F–H)	30	—	—	33
Total	8	80	8	8	104

TABLE 2. *First trimester initial zidovudine exposure: fetal abnormalities*

Patient	Exposure period during pregnancy: dose, indication	Outcome
A	Oral dose. Unknown × 4 weeks. HIV—1. | ——— | ---------- *	Multiple minor anomalies including low-set ears, retroganthia, hirsute, triangular face, blue sclera, prominent sacral dimple. Chromosomal analysis normal.
B	Oral dose. 750 mg/day × 8 weeks. HIV-1 with Pneumocystis carinii pneumonia. Amphotericin and Trimethoprim Sulpha-methoxazole. | ——— | ---------- *	Multiple minor anomalies, not specified but chromosomal analysis was normal.
C	Oral dose. 1200 mg/day × 12 weeks. HIV-1. | ——— | ---------- *	Extra digits on both hands, hare lip (central), and cleft palate.
D	Oral dose. 500 mg/day throughout. HIV-2. | ——— *	Fetal alcohol syndrome.

LMP 1 2 3 4 5 6 7 8 9 10 11 12 13 26 40
1st trimester (weeks) 2nd trimester 3rd trimester

Key: ----, gestational period following exposure; | ——— |, exposure period. * Time at which pregnancy ended.
LMP, last menstrual period.

sutism, triangular facies with blue sclera, hyperpigmented skin macules, and prominent sacral dimple. Chromosomal analysis was normal. Following discharge, both infants were monitored and subsequently, at 6 and 9 months, respectively, developed AIDS. In these infants HIV-1 virus was cultured from the blood samples.

Case C

Spontaneous labor at 36 weeks' gestation resulted in normal delivery of a female infant noted to have extra digits on both hands. There was no similar family history.

Case D

A pediatric diagnosis of fetal alcohol syndrome was made, although maternal alcohol intake could not be determined.

Second Trimester

Case E

A male infant born at 38 weeks had an asymptomatic atrial septal defect and pectus excavatum. Clinical AIDS was diagnosed at 3 months of age and the infant died within a further 2 months (Table 3).

TABLE 3. *Second trimester initial zidovudine exposure: fetal abnormalities*

Patient	Exposure period during pregnancy: dose, indication	Outcome
E	..... | Oral dose. 1200 mg/day × 23 weeks. AIDS. + Pentamidine aerosol. ——— | *	Asymptomatic. Atrial septal defect with pectus excavatum. Chromosomal studies normal.

LMP 13 14 15 16 17 18 19 20 21 22 23 24 25 26 40
1st trimester 2nd trimester (weeks) 3rd trimester

Key: ····· , gestational period prior to exposure; | ——— |, exposure period.
* Time at which pregnancy ended.

Third Trimester

Case F

A male infant born at 37 weeks had microcephaly and chorioretinitis. The mother was an intravenous drug abuser (IVA). An antenatal diagnosis of toxoplasmosis Gondii infection was also made and treated (Table 4).

Case G

A male infant born at 37 weeks had pectus excavatum, a deformity most likely to develop in the second or third trimester (4). Chromosomal studies were normal. The mother also had a history of schizophrenia with antidepressant use.

Case H

A female infant was born at 38 weeks with albinism and congenital ptosis following a diagnosis of oligohydramnios and intrauterine growth retardation.

DISCUSSION

While there are disadvantages to any retrospective study such as this, in a rapidly developing area of knowledge analysis of "what is" may have a valuable function which is not initially apparent. This is well illustrated by the area of HIV-1 infection in pregnancy and the occurrence of vertical transmission. As the number of AIDS cases increases, more women of childbearing age are known to be HIV-infected. This represents a growing potential for affected pregnancies and resultant at-risk neonates and infants. Given that the estimates of the risk of vertical transmission have declined each year, even the lower current figures reported of 15–35% (5,6) represent a major ethical problem in therapeutics (7).

The majority of fetuses will not be infected with HIV-1 in utero, and administration of a potentially fetotoxic drug to a symptomatic or an asymptomatic pregnant mother requires careful consideration. Put simply, from a fetal viewpoint the risk of intervention needs to be less than the risk of vertical transmission. This also requires evaluation in light of the concurrent maternal risks and clinical condition. Use of zidovudine in this population will probably increase, even without sufficient data concerning the safety or efficacy of this drug during pregnancy.

Teratogenic potential of Zidovudine in humans is still unknown. While animal studies have shown a direct toxic effect on mouse embryos, high-dose administration to pregnant rats and rabbits reportedly failed to demonstrate any fetal effects (1). However, the potential for cross-species differences tragically exemplified by thalidomide cannot be for-

TABLE 4. *Third trimester initial zidovudine exposure: fetal abnormalities*

Patient	Exposure period during pregnancy: dose, indication	Outcome
F	Oral dose. 140 mg I/V. 1,000 mg/day × last 70 days. 2 doses during labor. HIV-1 (IVA). ····· | ——— |[a] +Isoniazid, Trimethoprim, Sulphadiazine, Sulpha-methoxazole.	*Toxoplasma gondii* infection diagnosed. Microcephaly, chronoretinitis.
G	Oral dose. 500 mg/day last 57 days. HIV-2. ····· | ——— |[a] Antidepressants.	Pectus excavatum. Chromosomal analysis normal.
H	Oral dose. 1,200 mg/day × 70 days. HIV-1 (IVA). ····· | ——— |[a] Aerosol Pentamidine, Methodone, Heroin, Diazepam.	Albinism with congenital ptosis. Growth-retarded infant with oligohydramnios.

LMP 13 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
1st trimester 2nd trimester 3rd Trimester (weeks)

Key: ····· , gestational period prior to exposure; \| ——— \|, exposure period.
[a] Time at which pregnancy ended.

gotten. Pharmacokinetic data for zidovudine reported recently (2,8) suggest similar peak plasma levels and drug elimination curves in both pregnant and nonpregnant adults, with umbilical and amniotic fluid drug levels comparable with maternal blood levels.

In view of the myelosuppression reported from both pediatric and adult trials, this suggests a potential for fetal myelosuppression, in addition to any possible direct teratogenic effect. However, a reported study of zidovudine use in 43 pregnant women (9) failed to show any major adverse effect, although at the same time detailing a number of minor abnormalities which may be associated with zidovudine use.

While care must be taken not to generalize from negative reports in limited numbers of exposed patients, the current report of 104 such cases does serve to emphasize the dilemma of drug use in pregnancy. First-trimester exposure during organogenesis is of major concern, and it is of note that the spontaneous pregnancy loss rate in this group was comparable to a generally accepted level of 15%. The abnormalities noted in four cases are unremarkable in this context. Cases exposed during the second and third trimesters include a case of congenital toxoplasmosis with characteristic microcephaly and chorioretinitis, two chromosomally normal cases with clinical pectus excavatum, and a case with oligohydramnios and intrauterine growth retardation.

Evaluating the etiology of congenital abnormalities is difficult because multiple confounding factors can influence pregnancy outcome. To assess the question "is there evidence to suggest that zidovudine is teratogenic" is compounded by the additional unique characteristics of the population involved. In this grouping the same elements that influence the outcome of pregnancies in the general population will be present together with at least four additional factors: (a) HIV infection, (b) opportunistic infections, (c) drugs used in both HIV-1 and opportunistic infections, and (d) substance abuse (cigarettes, alcohol, illicit drugs).

In reviewing the frequency of birth defects in this population we noted eight birth defects (10%) out of 80 live births. A baseline risk of birth defects in the general population meeting the CDC criteria is 2–3% of live births, although the CDC uses retrospective record review. When prospective methods are used, rates as high as 5–7% have been reported (10). We currently do not have information on the expected proportion of birth defects in the HIV-infected population, which would be a more appropriate comparison group. Also, sample size remains too small for formal comparisons of the frequency of overall or specific birth defects.

Of the birth defects recognized, in only two cases was the timing of the exposure to zidovudine potentially relevant for the specific defects. In these cases (A and B, Table 2) the infants were born with multiple minor abnormalities.

It is of some interest that in two cases whose mothers began zidovudine in the second and third trimester, respectively, the infants were born with pectus excavatum (4). This anomaly is relatively rare as a congenital manifestation and usually occurs secondary to respiratory disorders in pediatrics (4). For the remaining abnormalities, one was clearly linked to toxoplasmosis infection. In the second, it is possible that maternal zidovudine therapy contributed to oligohydramnios and growth retardation (9), while for the third, no clear attribution could be made.

In summary, these data are reassuring in that no increase in or pattern of fetal abnormality could be attributed directly to maternal antenatal zidovudine exposure at all gestations. This is in agreement with two previous reports among smaller numbers (9,11). These cumulative data provide useful information for physicians who must counsel patients about zidovudine use in pregnancy in the context of maternal benefits versus uncertain fetal teratogenic risks. Any concern of possible long-term developmental defects following maternal zidovudine therapy and fetal exposure must await continuing follow-up.

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AIDS-defining opportunistic illnesses in US patients, 1994–2007: a cohort study

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Objectives: To assess the incidence and spectrum of AIDS defining opportunistic illnesses in the highly active antiretroviral therapy (cART) era.

Design: A prospective cohort study of 8070 participants in the HIV Outpatient Study at 12 U.S. HIV clinics.

Methods: We calculated incidence rates per 1000 person years of observation for the first opportunistic infection, first opportunistic malignancy, and first occurrence of each individual opportunistic illness during 1994 2007. Using stratified Poisson regression models, and adjusting for sex, race, and HIV risk category, we modeled annual percentage changes in opportunistic illness incidence rates by calendar period.

Results: Eight thousand and seventy patients (baseline median age 38 years; median CD4 cell count 298 cells/μl) experienced 2027 incident opportunistic illnesses during a median of 2.9 years of observation. During 1994 1997, 1998 2002, and 2003 2007, respectively, rates of opportunistic infections (per 1000 person years) were 89.0, 25.2 and 13.3 and rates of opportunistic malignancies were 23.4, 5.8 and 3.0 (P for trend <0.001 for both). Opportunistic illness rate decreases were similar for the subset of patients receiving cART. During 2003 2007, there were no significant changes in annual rates of opportunistic infections or opportunistic malignancies; the leading opportunistic illnesses (rate per 1000 person years) were esophageal candidiasis (5.2), *Pneumocystis* pneumonia (3.9), cervical cancer (3.5), *Mycobacterium avium* complex infection (2.5), and cytomegalovirus disease (1.8); 36% of opportunistic illness events occurred at CD4 cell counts at least 200 cells/μl.

Conclusions: Opportunistic illness rates declined precipitously after introduction of cART and stabilized at low levels during 2003 2007. In this contemporary cART era, a third of opportunistic illnesses were diagnosed at CD4 cell counts at least 200 cells/μl.

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Introduction

With the advent of highly active combination anti retroviral therapy (cART) in the mid 1990s and routine use of antimicrobial prophylaxis, the rates of AIDS defining opportunistic illnesses among HIV infected adults [1–9] and children [10,11] have declined dramatically in the US and other industrialized countries.

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Nonetheless, opportunistic illnesses remain a leading cause of hospitalization [12–14] and death [15–20] among HIV infected persons in these settings; late HIV diagnoses and acute opportunistic illnesses at first presentation to care remain common [21,22].

There have been few comprehensive studies of incidence of opportunistic infections and malignancies in the last decade in North America or Europe [7–9]. Although chronic non AIDS defining conditions are key determinants of morbidity and mortality on cART [15,23], opportunistic illnesses remain important markers of HIV disease progression; it is useful to monitor whether opportunistic illness rates have continued to fall or have stabilized, and whether the spectrum of opportunistic illness diagnoses has changed over time, so to inform HIV treatment guidelines, including thresholds for opportunistic illness prophylaxis initiation. Opportunistic illness rates could increase if more patients experience cART failure, if remaining therapeutic drug options are exhausted, or if HIV disease progresses for other reasons [24]. Studies suggest that some opportunistic illnesses [25,26], notably Kaposi's sarcoma [27], are occurring at higher than expected $CD4^+$ T lymphocyte cell counts (CD4 cell counts) among otherwise healthy virally suppressed patients; however, Kaposi's sarcoma at CD4 cell counts at least 200 has been documented both before and after the introduction of cART [20,28,29].

We examined the rates and patterns of opportunistic illness occurrence, and CD4 cell counts at opportunistic illness diagnoses, among HIV Outpatient Study (HOPS) patients seen during 1994–2007.

Methods

The HIV Outpatient Study

The HOPS is an ongoing, prospective cohort study of HIV infected patients since 1993 [1]. The study protocol is approved annually by each participating clinic's institutional review board. All study participants provide written, informed consent. HOPS clinicians have extensive experience treating HIV infected patients. Trained staff abstract data (including treatments, diagnoses, and laboratory values) from outpatient medical records at each visit, as well as hospitalizations and deaths. These data are compiled centrally, and reviewed and edited before analyses.

Study population

We analyzed data from 8070 HOPS participants who were seen at least twice from 1 January 1994 to 31 December 2007, using HOPS data updated as of 30 June 2008. We excluded patients seen only once because we could not define observation time for such patients to compute opportunistic illness incidence rates. The study sites included 12 clinics (university based, public, and private) in ten US cities: Tampa, Florida; Washington, DC; Denver, Colorado (two sites); Atlanta, Georgia; Portland, Oregon; San Leandro, California; Chicago, Illinois (two sites); Stony Brook, New York; Philadelphia, Pennsylvania; Oakland, California.

Definitions

Start of observation (baseline) was 1/1/1994 or first HOPS visit thereafter. We defined three time periods for analyses: 1994–1997 (pre cART), 1998–2002 (early cART), and 2003–2007 (contemporary cART). We used accepted definitions for medications comprising cART and *Pneumocystis* pneumonia (PCP) prophylaxis and *Mycobacterium avium* complex (MAC) prophylaxis [30], as detailed in Appendix 2 (http://links.lww.com/QAD/A27). We studied all AIDS defining opportunistic illnesses using the CDC revised 1993 AIDS case definition [31], except recurrent pneumonia (because we were unable to distinguish viral from bacterial pneumonia in the HOPS database), *Salmonella* septicemia (because there was only one case), and HIV wasting syndrome (because of lack of a standardized case definition and diagnostic specificity across HOPS clinics). Outcomes of interest were opportunistic illness diagnoses made during HOPS clinic visits or hospitalizations, using supporting laboratory or diagnostic tests when required or available. We excluded the small fraction of opportunistic illnesses newly diagnosed at death in primary analyses. We considered Kaposi's sarcoma, non Hodgkin's lymphoma, CNS lymphoma and cervical cancer as opportunistic malignancies. All other AIDS defining opportunistic illnesses were classified as opportunistic infections. We considered as major opportunistic illnesses those for which at least 15 events had occurred during the contemporary cART period.

Entry values for CD4 cell count and HIV viral load for each time period were those measured within 6 months prior through 3 months after the beginning of observation in the period. CD4 cell count at opportunistic illness diagnosis was the closest CD4 cell count within 6 months prior through 3 months after the date of opportunistic illness diagnosis.

Determination of opportunistic illness rates

Annual rates of first opportunistic illness events (1994–2007) were calculated for patients with at least one HOPS contact in a given year and without the specific opportunistic illness previously. Start of observation for each year was 1 January (if the patient was already enrolled in the HOPS) or the date of first HOPS visit during that year. For each opportunistic illness, end of observation was the earlier of 31 December of that year, date of incident opportunistic illness diagnosis (patient removed from the risk set), or last (alive) HOPS contact. In an analogous fashion, we calculated opportunistic illness incidence rates in each calendar period.

Incidence rate calculations for each opportunistic illness excluded patients with a history of that opportunistic illness at the start of observation, and considered patients no longer at risk for that opportunistic illness (censored observation time) after it was diagnosed in follow up. Likewise, we calculated rates of any first opportunistic infection and any first opportunistic malignancy, after excluding patients with the history of either, respectively, from the analyses. Thus persons with a prior diagnosis of a given opportunistic illness were excluded from analyses of incidence of that opportunistic illness, but are included in the analyses of incidence of another opportunistic illness (also see Appendix 2, http://links.lww.com/QAD/A27).

Determination of opportunistic illness prophylaxis rates

We examined the annual percentage of eligible patients who were prophylaxed for at least 30 days for PCP or MAC during 1994–2007. Patients were considered eligible for primary PCP prophylaxis when their CD4 cell count was below 200 cells/μl and for MAC prophylaxis when their CD4 cell count was below 50 cells/μl [30]. We restricted analyses to patients with a documented CD4 cell count below the eligible threshold in a given year and with at least 90 days of subsequent observation to ensure adequate opportunity to have received prophylaxis.

Statistical methods

We calculated annual incidence rates per 1000 person years with 95% confidence intervals (CIs) using a Poisson distribution for the first opportunistic infection, first opportunistic malignancy, and first occurrence of each individual opportunistic illness. Using stratified (by period) multivariable Poisson regression, we modeled annual percentage changes in opportunistic illness incidence rates within the three time periods for major opportunistic illnesses. We first explored with univariate regression models associations between major incident opportunistic illnesses and age, sex, race/ethnicity and HIV risk category. In the final multivariable models, we adjusted for sex, race, and HIV risk category, but omitted age as it was not associated with opportunistic illness rates. We further evaluated factors predictive of incident opportunistic illnesses in the contemporary cART era by considering entry CD4 cell count, HIV viral load, and type of health insurance as independent variables. We report rate ratios and 95% CIs. Finally, we assessed changes in CD4 cell count distribution at opportunistic illness diagnosis, by calendar period, using the nonparametric Jonckheere–Terpstra test. We performed analyses using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Of 8517 patients who were seen in the HOPS between 1 January 1994 and 31 December 2007, we excluded 447 (5%) who were seen only once and for whom follow up could not be defined. Excluded patients were not statistically different from included patients with respect to sex and age distribution, history of AIDS at first HOPS visit (45 vs. 48%) or baseline median CD4 cell count (273 vs. 303 cells/μl), but were significantly more likely to be non white (62 vs. 43%) and have public insurance (59 vs. 37%) at HOPS entry.

Of 8070 patients analyzed (median age at baseline 38 years; median CD4 cell count 298 cells/μl), 81% were men, 57% white, 59% men who had sex with men (MSM) and 13% injection drug users (IDUs) (Table 1). During 1994–2007, the percentages of women, non white persons, and persons with heterosexual risk for HIV infection increased, as did median age. The median duration of follow up in the study was 2.9 years [interquartile range (IQR) 1.1–6.9]. The percentage of patients who received cART (≥1 day) within each time period increased from 56% during 1994–1997 to 88% during 2003–2007, with the corresponding increase in the percentage of total observation time on cART from 28.7 to 78.5%, respectively (Table 1). The percentage of eligible patients (CD4 cell count <200 cells/μl) who received primary PCP prophylaxis each year (annual range 75.8–93.7%) decreased from 93.7% in 1994 to 78.6% during 2007 (univariate $P < 0.001$). The percentage of eligible patients (CD4 cell count <50 cells/μl) who received primary MAC prophylaxis each year (annual range 53.2–74.3%) did not change (53.2% in 1994 to 66.7% in 2007, univariate $P = 0.25$).

Trends in rates of opportunistic illnesses

Seven opportunistic illnesses met our definition for major incident opportunistic illnesses (>15 cases in the contemporary cART era): esophageal candidiasis ($n = 67$), PCP ($n = 46$), disseminated MAC ($n = 32$), cytomegalovirus (CMV) disease, including retinitis ($n = 23$), Kaposi's sarcoma ($n = 16$), non Hodgkin's lymphoma [(NHL) $n = 21$], and HIV encephalopathy ($n = 18$).

We observed 2027 incident opportunistic illness events during 35 236 person years of observation. In analyses of incidence of first opportunistic infection and first opportunistic malignancy, we excluded, respectively, 1313 and 282 patients with a prior diagnosis of these events at baseline. Among seven major individual opportunistic illnesses, the following were excluded from the analyses of that opportunistic illness: 136 patients with a history of esophageal candidiasis, 760 with PCP, 148 with MAC, 179 with CMV disease; 228 with Kaposi's sarcoma, 46 with NHL and 27 with HIV encephalopathy.

All opportunistic illness incidence rates fell during 1994–2007 (Table 2), most notably during 1994–1997, coinciding with cART introduction (Fig. 1a, b). Overall opportunistic illness incidence rates (per 1000 person years) were 92.4 (95% CI 84.5–100.8) in 1994–1997,

Table 1. Characteristics of patients included in analyses of AIDS-defining opportunistic illnesses incidence rates, the HIV Outpatient Study, 1994–2007 (*N* = 8070).

Characteristic	1994–1997 (*N* = 4231) PY = 7216	1998–2002 (*N* = 4789) PY = 13 736	2003–2007 (*N* = 4314) PY = 13 706	Overall[a] (*N* = 8070) PY = 35 236
	n (%)	*n* (%)	*n* (%)	*n* (%)
Median age[b] (years)	37	39	42	38
Male sex	3496 (83)	3774 (79)	3409 (79)	6516 (81)
Race/ethnicity				
Non Hispanic white	2697 (64)	2613 (55)	2324 (54)	4562 (57)
Non Hispanic black	1051 (25)	1518 (32)	1350 (31)	2424 (30)
Hispanic	376 (9)	521 (11)	512 (12)	842 (10)
Other/unknown	107 (3)	137 (3)	128 (3)	242 (3)
HIV risk group				
MSM	2577 (61)	2720 (57)	2548 (59)	4729 (59)
Heterosexual	770 (18)	1210 (25)	1108 (26)	1822 (23)
IDU	661 (16)	640 (13)	442 (10)	1087 (13)
Other/unknown	223 (6)	219 (4)	216 (5)	432 (5)
Insurance payer[b]				
Private	1672 (40)	2494 (52)	2435 (56)	3728 (46)
Public	1521 (36)	1857 (39)	1605 (37)	2963 (37)
Other/unknown	1038 (25)	438 (9)	274 (6)	1379 (17)
CD4 cell count[b] (cells/μl)				
0–49	621 (15)	430 (9)	218 (5)	1040 (13)
50–199	859 (20)	801 (17)	528 (12)	1508 (19)
200–349	785 (19)	957 (20)	761 (18)	1517 (19)
350+	1329 (31)	2146 (45)	2376 (55)	3074 (38)
Unknown	637 (15)	455 (10)	431 (10)	931 (12)
Median	257 (*n* = 3594)	345 (*n* = 4334)	421 (*n* = 3885)	298 (*n* = 7141)
HIV viral load[b] (copies/ml)				
<1000	336 (8)	1919 (40)	2101 (49)	1693 (21)
1000–99 999	734 (17)	1774 (37)	1304 (30)	2175 (27)
100 000+	340 (8)	628 (13)	454 (11)	1048 (13)
Unknown	2821 (67)	468 (10)	455 (11)	3154 (39)
Median	13 263 (*n* = 1410)	2169 (*n* = 4321)	536 (*n* = 3859)	8150 (*n* = 4916)
Used cART[c] in the period	2351 (56)	4152 (87)	3787 (88)	6027 (75)
PY observation (%)				
Total	7216	13 736	13 706	35 236
No ART	1838 (25.5)	2575 (18.7)	2469 (18.0)	6977 (19.8)
NoncART	3307 (45.8)	940 (6.8)	475 (3.5)	4745 (13.5)
cART	2071 (28.7)	10 220 (74.4)	10 761 (78.5)	23 514 (66.7)
Median PY observed (IQR)	1.3 (0.6–2.8)	2.8 (1.1–5.0)	3.5 (1.6–5.0)	2.9 (1.1–6.9)

ART, antiretroviral therapy; cART, highly active combination ART; IDU, injection drug user; IQR, interquartile range; MSM, man (or men) who have sex with men; PY, person years.
[a]Patients could be included in more than one period. Overall column shows unique patients for whom data are summarized from the first available period.
[b]Measured as of beginning of observation in the period (closest value, within 6 months prior to 3 months after).
[c]Received cART at least 1 day within the time period. See text for definition of cART regimens. Non cART regimens included all mono, dual and triple antiretroviral combination regimens which did not meet criteria for cART.

29.6 (95% CI 26.4–33.1) in 1998–2002, and 16.6 (95% CI 14.2–19.3) in 2003–2007. In crude analyses, rates of first opportunistic infection and first opportunistic malignancy (Kaposi's sarcoma, NHL and CNS lymphoma) in the contemporary cART period were each significantly lower than in earlier periods (Table 2). Rates of first opportunistic infection and first opportunistic malignancy (per 1000 person years) decreased for all patients and those receiving cART in a similar fashion (Fig. 2).

Opportunistic illnesses with the highest incidence rates during 1994–1997 were CMV disease, including retinitis (33.0), PCP (29.9), MAC (26.9), esophageal candidiasis (21.6), and Kaposi's sarcoma (16.4); during 2003–2007 they were esophageal candidiasis (5.2), PCP (3.9), cervical cancer (3.5), MAC (2.5), and CMV disease (1.8) (Table 2). Thus, opportunistic infections with the highest incidence rates in the first period remained among the most frequently observed in the last period.

In multivariable Poisson regression models adjusted for sex, race and HIV risk category (variables associated with opportunistic illness events in crude analyses; Supplemental online Table A, http://links.lww.com/QAD/A28), there were significant annual percentage reductions during 1994–1997 in incidence of first opportunistic infection, first opportunistic malignancy, and each major individual opportunistic illness, except for HIV encephalopathy and esophageal candidiasis (Table 3). During 1998–2002, declines continued, but were of lesser magnitude, for

Table 2. Absolute numbers and rates per 1000 person-years of incident AIDS-defining opportunistic illnesses, the HIV Outpatient Cohort Study, 1994–2007.

	1994–1997		1998–2002		2003–2007	
	N	Rate (95% CI)	*n*	Rate (95% CI)	*n*	Rate (95% CI)
Any opportunistic illness	505	92.4 (84.5–100.8)	303	29.6 (26.4–33.1)	170	16.6 (14.2–19.3)
Person years		5465		10 236		10 241
Opportunistic infection[a]	486	89.0 (81.3–97.3)	258	25.2 (22.2–28.5)	139	13.3 (11.2–15.7)
Person years		5459		10 240		10 447
Opportunistic malignancy[b]	153	23.4 (19.8–27.4)	73	5.8 (4.6–7.3)	38	3.0 (2.1–4.1)
Person years		6536		12 552		12 699
Cytomegalovirus disease, all	219	33.0 (28.6–37.7)	60	4.7 (3.6–6.0)	23	1.8 (1.1–2.7)
CMV retinitis	164	24.4 (20.8–28.4)	42	3.2 (2.3–4.4)	5	0.4 (0.1–0.9)
CMV other	103	15.1 (12.3–18.3)	37	2.9 (2.0–3.9)	20	1.5 (0.9–2.4)
Pneumocystis pneumonia	185	29.9 (25.7–34.5)	90	7.7 (6.2–9.5)	46	3.9 (2.9–5.2)
Mycobacterium avium complex infection	181	26.9 (23.1–31.1)	79	6.2 (4.9–7.7)	32	2.5 (1.7–3.5)
Esophageal candidiasis	145	21.6 (18.2–25.4)	120	9.5 (7.9–11.4)	67	5.2 (4.1–6.7)
Kaposi's sarcoma	108	16.4 (13.4–19.7)	37	2.9 (2.0–4.0)	16	1.2 (0.7–2.0)
Cryptosporidiosis	50	7.3 (5.4–9.6)	26	2.0 (1.3–2.9)	10	0.8 (0.4–1.4)
Non Hodgkin's lymphoma	38	5.5 (3.9–7.6)	37	2.8 (2.0–3.9)	21	1.6 (1.0–2.4)
HIV encephalopathy	37	5.4 (3.8–7.4)	24	1.8 (1.2–2.7)	18	1.4 (0.8–2.2)
Mycobacterium tuberculosis infection	34	5.0 (3.4–7.0)	24	1.9 (1.2–2.8)	11	0.8 (0.4–1.5)
Toxoplasmosis	28	4.1 (2.7–5.9)	17	1.3 (0.8–2.1)	6	0.5 (0.2–1.0)
Cervical cancer[c]	4	3.5 (1.0–8.9)	2	0.7 (0.1–2.7)	10	3.5 (1.7–6.5)
CNS lymphoma	21	3.0 (1.9–4.6)	5	0.4 (0.1–0.9)	3	0.2 (0.0–0.7)
PML	19	2.7 (1.6–4.3)	10	0.8 (0.4–1.4)	9	0.7 (0.3–1.3)
Cryptococcosis	18	2.6 (1.5–4.1)	15	1.2 (0.6–1.9)	11	0.8 (0.4–1.5)
Atypical mycobacteriosis[d]	17	2.5 (1.4–3.9)	12	0.9 (0.5–1.6)	2	0.2 (0.0–0.5)
Pulmonary candidiasis	14	2.0 (1.1–3.4)	11	0.8 (0.4–1.5)	2	0.2 (0.0–0.5)
Chronic herpes simplex disease	11	1.6 (0.8–2.8)	15	1.1 (0.6–1.9)	13	1.0 (0.5–1.7)
Histoplasmosis	6	0.9 (0.3–1.9)	8	0.6 (0.3–1.2)	0	0.0 (0.0–0.3)
Total events	1135		592		300	

CMV, cytomegalovirus; CNS, central nervous system; OI, opportunistic illness; PML, progressive multifocal leukoencephalopathy.
[a]Includes all AIDS defining opportunistic infections, except recurrent pneumonia (because unable to distinguish viral from bacterial pneumonia in the database) and *Salmonella* septicemia (because only one case).
[b]Includes Kaposi's sarcoma, Non Hodgkin's lymphoma and CNS lymphoma (but not cervical cancer as only pertaining to women).
[c]Incidence calculated among women.
[d]Includes *M. kansasii*, *M. fortuitum*, and *M. abscessus*.

opportunistic infections overall and individual opportunistic illnesses: CMV, PCP, MAC and Kaposi's sarcoma. During 2003–2007, only MAC rates continued to fall; no opportunistic illness rates increased. Adjustment for current CD4 cell count, a critical determinant of opportunistic illness risk and also a laboratory marker affected by cART use (and thus associated with calendar period), tended to attenuate the percentage reductions in opportunistic illness rates over time (see footnote to Table 3).

Absolute rates and trends in opportunistic illness rates also did not change substantially when opportunistic illnesses first diagnosed at death (within 60 days of last patient contact with the HOPS) were included in analyses (only 33 events or 1.6% of all opportunistic illness events, data not shown). Results were also not meaningfully affected by analyzing all (first and repeat) opportunistic illness events for two of the major opportunistic illnesses that frequently recur: PCP and esophageal candidiasis (data not shown).

Risk factors for increased incidence of opportunistic illnesses

In univariate analyses, incidence rates of first opportunistic infection were generally higher for women (vs. men); non whites (vs. whites); heterosexuals and IDUs (vs. MSM); publicly insured (vs. privately insured); and persons with lower CD4 cell counts, lower nadir CD4 cell counts and higher HIV viral loads at the start of observation in each analysis period (Supplemental online Table A, http://links.lww.com/QAD/A28). For opportunistic malignancies (excluding cervical cancer, which only affects women), incidence rates were higher for men in the first period, MSM (vs. heterosexuals) and persons of white race (vs. non white) in the second period, and were generally higher in patients with lower CD4 cell counts, lower nadir CD4 cell counts, and higher HIV viral loads at the start of each period (i.e., entry measurements).

In multivariable analyses of the contemporary cART period (2003–2007), factors independently associated with higher opportunistic infection rates included public insurance, lower entry CD4 cell counts and higher entry HIV viral load (Supplemental online Table B, http://links.lww.com/QAD/A29). Viremia was associated most strongly with higher incidence of opportunistic infections among patients with entry CD4 cell count above 200 cells/μl: for $\log_{10}$ viral load 3–4 vs. less than 3 [adjusted relative risk (RR), 95% CI 2.17, 1.50–3.12]; for

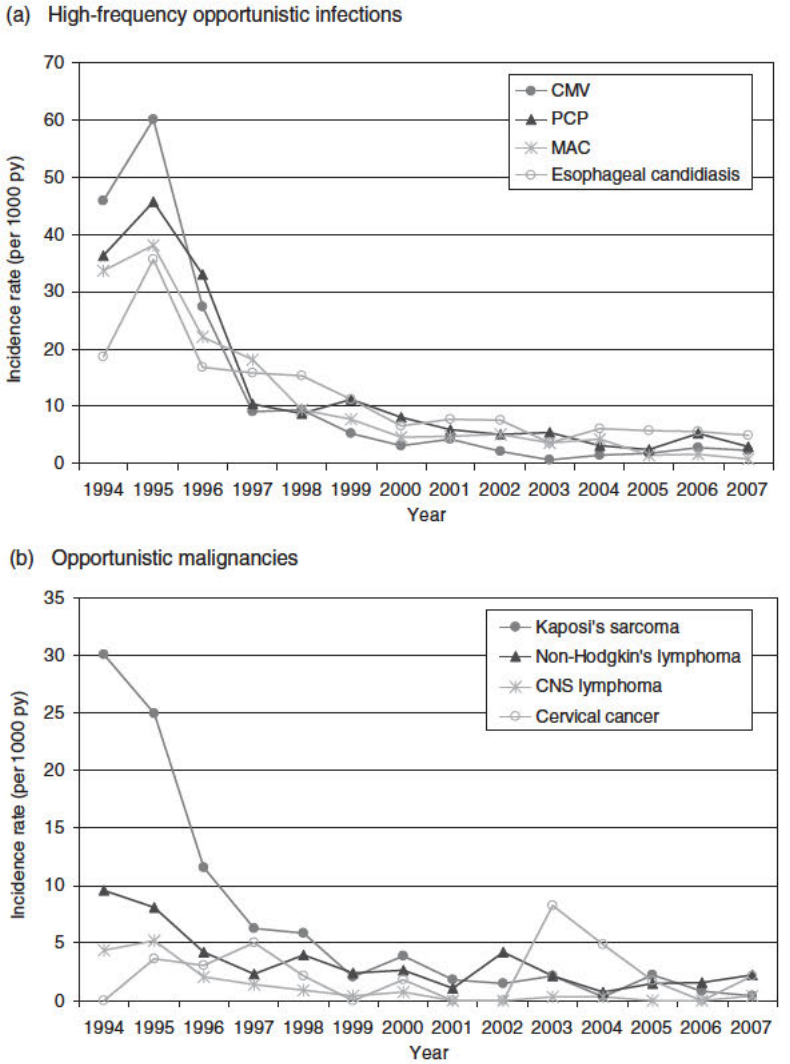

Fig. 1. Incidence of AIDS-defining opportunistic illnesses, the HIV Outpatient Study, 1994 2007. (a) High-frequency opportunistic infections; (b) opportunistic malignancies.

$\log_{10}$ viral load at least 5 vs. less than 3 (5.61, 3.48–9.04). Higher HIV viral load, but not lower CD4 cell count, was independently associated with higher rates of opportu nistic malignancy (Supplemental online Table B, http://links.lww.com/QAD/A29). When nadir CD4 cell count, instead of entry CD4 cell count, was included in these models, the findings were not markedly different, although associations with CD4 cell count were weaker (data not shown).

Finally, when we limited analyses to patients who were on cART as they entered contemporary cART period, lower CD4 cell count (cells/μl) and higher viral load (copies/ml) remained independently associated with increased rates of opportunistic infections: for CD4 cell count 50–199 vs. at least 200 (RR, 95% CI 2.70, 1.41–5.17), for CD4 cell count below 50 vs. at least 200 (11.12, 5.00–24.74); for $\log_{10}$ viral load 3–4 vs. less than 3 (3.37, 1.73–6.52); for $\log_{10}$ viral load at least 5 vs. less than 3 (3.02, 1.29–7.08). For malignant opportunistic illnesses, only $\log_{10}$ HIV viral load at least 5 vs. less than 3 (4.76, 1.65–13.72) was independently associated with higher event rates on cART.

Trends in CD4 cell counts at opportunistic illness diagnosis

Of all opportunistic illness events with documented CD4 cell counts at diagnosis (measured a median of 28 days after diagnosis, IQR 8–62 days), 13% of events occurred at CD4 cell counts at least 200 cells/μl during 1994–1997 (122/929), 22% during 1998–2002 (116/539), and 35% during 2003–2007 (94/268), univariate test for trend $P < 0.0001$. These findings corresponded with increases in the percentage of all active HOPS patients who had CD4 cell counts at least 200 cells/μl over time (see Table 1).

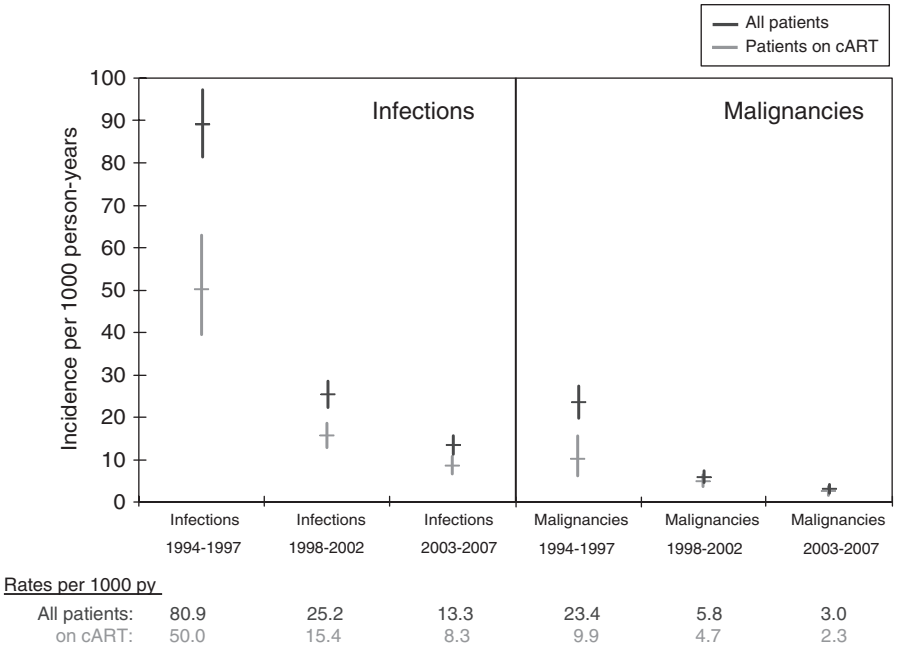

Fig. 2. Incidence rates (95% CIs) of first opportunistic infection and first opportunistic malignancy among all patients observed and among those patients followed on cART, by analysis period, HIV Outpatient Study, 1994 2007.

Among major opportunistic illnesses, CD4 cell counts at opportunistic illness diagnosis increased significantly ($P < 0.05$) over time for CMV, esophageal candidiasis, Kaposi's sarcoma, NHL, and HIV encephalopathy (Table 4). Except for NHL and CMV, these findings persisted in analyses limited to opportunistic illnesses diagnosed among patients receiving cART. Notably, the range of CD4 cell count (5th–95th percentile) at diagnosis was wide for many opportunistic illnesses both before and after the widespread cART use (Table 4).

Opportunistic illnesses in the contemporary combination antiretroviral therapy period

Among 300 total opportunistic illness diagnoses (250 opportunistic infections, 50 opportunistic malignancies) during 2003–2007 (Table 2), 32 (11%) were among antiretroviral naive persons and 42 (14%) among patients diagnosed with HIV infection during the previous year. Of 268 opportunistic illnesses with CD4 cell counts recorded at diagnosis, 94 (35%) occurred at CD4 cell counts at least 200 cells/μl (including 19 esophageal

Table 3. Adjusted annual changes in incidence rates of select AIDS-defining opportunistic illnesses, the HIV Outpatient Study, 1994–2007.

Category	Adjusted estimates of annual percentage change in incidence rate during period (95% CI)[a]		
	1994 1997	1998 2002	2003 2007
Opportunistic infection[b]	27 (36, 18)	15 (23, 7)	6 (18, 7)
Opportunistic malignancy[c]	37 (44, 28)	12 (29, 9)	2 (21, 20)
Cytomegalovirus disease, all	37 (50, 21)	28 (43, 10)	29 (3, 72)
Pneumocystis pneumonia	30 (41, 17)	16 (27, 3)	8 (24, 13)
Mycobacterium avium complex	23 (35, 10)	17 (28, 5)	29 (47, 6)
Kaposi's sarcoma	39 (47, 29)	27 (42, 7)	22 (44, 10)
Non Hodgkin's lymphoma	37 (50, 19)	1 (25, 30)	6 (21, 42)
HIV encephalopathy	13 (35, 16)	1 (25, 29)	18 (15, 65)
Esophageal candidiasis	18 (33, 1)	18 (27, 8)	6 (13, 30)

OI, opportunistic illness.
[a]Estimates derived from the multivariable Poisson regression models for the OIs which had at least 15 events in the last observation period. The estimates are adjusted for sex, race, and HIV risk category. Further adjustment for current $CD4^+$ cell count (closest to the start of observation in each year) tended to attenuate the estimated changes in rates of OIs over time; for example, for opportunistic infections, the estimates of annual percentage changes in incidence rates (95% CIs) for the three periods, respectively, were: 23 (30, 15), 12 (21, 3), and 2 (14, 11); and for opportunistic malignancies they were: 34 (44, 23), 7 (21, 9), and 0 (16, 20).
[b]Includes all AIDS defining opportunistic infections, except recurrent pneumonia (because unable to distinguish viral from bacterial pneumonia in the database) and *Salmonella* septicemia (because only one case).
[c]Includes Kaposi's sarcoma, Non Hodgkin's lymphoma and CNS lymphoma (but not cervical cancer as only pertaining to women).

Table 4. CD4 cell counts at opportunistic illness diagnoses, by calendar period, HIV Outpatient Study, 1994–2007.

	Median CD4 cell count, cells/μl (5th 95th percentile)									
	1994 1997			1998 2002			2003 2007			
	N^b	*n*	Median	*N*	*n*	Median	*N*	*n*	Median	P^a trend
Cytomegalovirus disease	219	172	20 (2 259)	60	57	23 (2 226)	23	20	61 (4.5 630)	0.02
	27	25	29 (7 419)	34	32	30.5 (4 226)	13	13	62 (6 697)	0.11
Pneumocystis pneumonia	185	164	55 (3 418)	90	82	48.5 (4 440)	46	41	93 (11 429)	0.44
	22	20	28 (1 419)	34	33	52 (4 440)	18	17	112 (7 528)	0.22
Mycobacterium avium complex infection	181	132	22 (1 150)	79	68	22.5 (1 227)	32	30	24 (1 266)	0.23
	27	19	13 (0 316)	34	29	43 (4 257)	17	16	57 (10 266)	0.47
Kaposi's sarcoma	108	97	38 (2 359)	37	35	98 (4 753)	16	14	273.5 (6 476)	<0.001
	12	11	25 (1 220)	16	16	109 (7 826)	9	8	201 (6 446)	0.01
Esophageal candidasis	145	123	43 (4 460)	120	109	46 (3 378)	67	63	100 (3 493)	0.02
	28	26	34 (1 465)	49	45	88 (3 361)	28	28	134.5 (7 319)	0.03
Non Hodgkin's lymphoma	38	30	73 (4 361)	37	35	219 (39 761)	21	19	250 (30 943)	<0.001
	5	5	96 (10 851)	23	22	182.5 (39 568)	13	11	243 (30 864)	0.25
HIV encephalopathy	37	30	43 (3 484)	24	22	208 (4 437)	18	17	223 (4 660)	0.02
	8	8	73 (3 240)	16	15	226 (4 499)	7	6	248 (74 653)	0.01

For each OI, the first row presents data for all cases, the second row presents data for the cases diagnosed among persons receiving cART. OI, opportunistic illness.
[a]Trend in CD4 cell counts by time period evaluated in univariate analyses by Jonckheere Terpstra nonparametric test.
[b]*N* is total number of patients diagnosed with an OI; n is number with available $CD4^+$ cell count at OI diagnosis.

candidiasis, 13 NHL, 11 PCP, nine HIV encephalopathy and eight Kaposi's sarcoma). Of the 94, 14% met AIDS criteria based on associated CD4% less than 14. Finally, among 259 opportunistic illness diagnoses with recorded CD4 cell counts and HIV viral loads at opportunistic illness diagnosis in this period, 45 (17%) were documented in patients with CD4 cell count at least 200 cells/μl and HIV viral load less than 1000 copies/ml; some of these events might have represented immune reconstitution inflammatory syndrome (IRIS) [30], an area of ongoing investigation in the HOPS [32].

Discussion

In our HOPS cohort, the incidence rates of opportunistic illnesses fell sharply during 1994–1997, either remained stable or declined more gradually during 1998–2002, and stabilized at low levels during 2003–2007. In general, opportunistic illnesses with the highest incidence in the pre cART era were also the most frequently diagnosed in the cART eras. In the contemporary cART era, opportunistic illnesses occurred predominately among antiretroviral experienced patients, and approximately one third occurred among persons with CD4 cell counts at least 200 cells/μl. Among patients on cART, opportunistic illness rates were significantly lower in 2003–2007 than 1998–2002, a finding consistent with improved potency and tolerability of newer cART regimens. However, higher HIV viral loads and lower CD4 cell counts remained associated with opportunistic illnesses among cART recipients; these factors could represent patients with more recent cART initiation, suboptimal responses to cART or medication nonadherence. The decreasing rates of PCP prophylaxis and fluctuations in rates of MAC prophylaxis over time raise the possibility that some opportunistic illnesses might have occurred because prophylaxis was not restarted promptly among cART experienced patients when their CD4 cell counts fell below the threshold for prophylaxis initiation, a phenomenon documented in other studies [33].

Although direct comparisons of our observed opportunistic illness rates to those reported from other cohorts are difficult because of multiple cross cohort differences, including degree of immunosuppression and differences in analytic methods, our absolute opportunistic illness rates were generally of the same magnitude as in other studies of opportunistic infections and malignancies both before and during cART era [2,8,9,34].

In addition to low CD4 cell counts and high HIV viral loads [3,35], certain demographic factors are associated with increased risk for specific opportunistic illnesses [2,3,9,26]: older age (e.g. for opportunistic malignancies [8]), male sex (e.g. for Kaposi's sarcoma [2]), and HIV risk (e.g. IDU and MSM for a variety of opportunistic infections [2,9]). In our adjusted analyses for 2003–2007, publicly insured HOPS patients were significantly more likely than privately insured patients to experience opportunistic infections (public insurance being associated with lower socioeconomic status and later entry to HIV care in the HOPS), and women were less likely than MSM to experience opportunistic malignancies (likely explained, at least in part, by higher rates of Kaposi's sarcoma among MSM and the exclusion of cervical cancer from consideration in this comparison). We did not detect significant differences in opportunistic illness

rates by race/ethnicity or age in the contemporary cART period, but our study population had a narrow age range (IQR 33–44 years). Having a CD4 cell count <50 cells/μl remained the strongest predictor of an incident opportunistic infection in that period, whereas an HIV viral load above 100 000 copies/ml (vs. <1000 copies/ml) was associated with increased risk for an opportunistic malignancy among all patients and those on cART.

The finding that median CD4 cell counts at opportunistic illness diagnosis for CMV, esophageal candidiasis, Kaposi's sarcoma, NHL and HIV encephalopathy have increased during 1994–2007 is intriguing. A considerable variability in CD4 cell counts at diagnosis of opportunistic illnesses has been documented before and after introduction of cART [9,20,26]. Two principal hypotheses exist to explain observed trends [4,26,34,36]. First, the increase in median CD4 cell count at diagnosis, for the few opportunistic illness events that still occur, is likely a reflection of increased CD4 cell counts of the entire HOPS population followed after introduction of cART (see Table 1). Second, cART associated immune restoration is functionally incomplete, particularly among persons who had experienced profound $CD4^+$ T cell depletion, resulting in opportunistic illness occurrence at higher CD4 cell counts.

Our study has several limitations. First, we analyzed chart abstracted data collected in the course of routine clinical care. Although most opportunistic infections present acutely and thereby bring affected patients into clinical care, some opportunistic illnesses might have gone undetected due to presentation and care provided outside of a HOPS facility, subclinical presentation or incomplete screening (e.g. cervical cancer); thus leading to potential underestimation of opportunistic illness incidence in our population. Conversely, overestimation of opportunistic illness incidence rates could have resulted from inadvertent inclusion of patients with opportunistic illness histories that were not documented in available medical records. Provider feedback and exploratory analyses suggest that the extent of this misclassification was modest and constant over time. Second, our study excludes recurrent bacterial pneumonia, a relatively frequent opportunistic illness [30], because differentiation between bacterial and viral pneumonia was often undocumented, and excludes HIV wasting syndrome, a condition lacking diagnostic specificity and standardized definition across HOPS clinics. Third, we performed an ecological analysis of trends rates of opportunistic illnesses, cART usage and opportunistic illness prophylaxis and therefore cannot draw causal inferences; however, it has been well established that cART and opportunistic illness prophylaxis reduce opportunistic illness rates [1,3,6,9,37]. Fourth, due to relatively small numbers of events, particularly in the contemporary cART period, we may have failed to detect some significant annual changes in individual opportunistic illness rates due to low statistical power. Finally, our findings are drawn from a convenience sample of patients receiving care at 12 select private and public HIV specialty US clinics, and are likely generalizable only to diagnosed patients in care in industrialized countries.

In conclusion, in our diverse cohort of HIV infected patients in the US, rates of the major AIDS defining opportunistic illnesses in the cART era have fallen 5–20 fold to approximately five cases per 1000 person years or less for each opportunistic illness, and have remained stable during 2003–2007. Opportunistic illnesses that predominated in the pre cART period have remained prominent in the contemporary cART period. Low CD4 count and high HIV viral load remain associated with incident opportunistic illnesses. However, a minority of patients have opportunistic illnesses diagnosed at higher than expected CD4 cell counts and possibly while virally suppressed. Healthcare providers need to maintain vigilance in looking for incident opportunistic illnesses, and ensure that all patients are appropriately screened for opportunistic malignancies and prescribed antimicrobial prophylaxis for opportunistic infections as recommended [30], to further reduce rates of AIDS and mortality [20] among contemporary HIV infected patients.

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Disclaimers: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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REVIEWING THE DATA–II

Fulfilling Koch's Postulates

A century ago, German bacteriologist Robert Koch devised a test for proving that a disease is caused by a specific microbe. That test, known as "Koch's postulates," has become a standard in medicine. Peter Duesberg claims HIV fails it. But some researchers think recent evidence suggests the virus does pass this test.

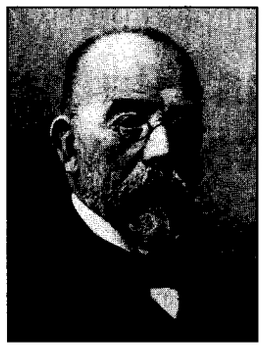
Koch maintained that for causation to be established, it must be possible to isolate the microbe from an organism that has come down with the disease. The microbe must then be given to a healthy host, where it causes the same disease; then the microbe must be isolated again. Until recently, many AIDS researchers agreed HIV had not satisfied Koch's postulates, largely because there is no good animal model for AIDS. But those researchers did not agree that because HIV didn't satisfy Koch's postulates, it wasn't the cause of AIDS. They pointed out that Koch's postulates have not been satisfied in many other diseases where the cause has been well established by other means.

But recently some leading AIDS researchers have stopped conceding that HIV doesn't satisfy Koch's postulates, as powerful new evidence emerged from tragic accidents: the infection of three laboratory workers with a pure, molecularly cloned strain of HIV. As reported at the 1993 international AIDS conference in Berlin by the National Cancer Institute's William Blattner and his colleagues, one of the three lab workers developed *Pneumocystis* pneumonia, an AIDS-defining disease, 68 months after showing evidence of infection. This lab worker had not received AZT (which Duesberg contends can cause AIDS), or any other anti-HIV drug, until 83 months after infection, when the patient had fewer than 50 CD4 cells, the key immune system cells destroyed by HIV. (A healthy person typically has a count of 600 to 1200 CD4s.)

Blattner reported that a second lab worker, who also received no anti-viral drugs, had 250 to 400 CD4s at 83 months. The third lab worker had CD4 counts of 200 to 500 at 25 months and had been given anti-virals. "These people have no other risk factors" for AIDS, such as illicit drug injection or homosexual sex, Blattner says.

Duesberg told *Science* that, in his view, the lab-worker data don't prove that HIV satisfies Koch's postulates. Two of the lab workers, he notes, did not have AIDS, but only a severe decline in CD4 counts. Duesberg did not directly address data on the one lab worker who has the AIDS-defining illness *Pneumocystis* pneumonia and therefore does have AIDS. Instead, Duesberg responded by asking why, if HIV causes AIDS, more HIV-positive people don't develop this AIDS-defining pneumonia within 5 years. (The average time between HIV infection and an AIDS-defining illness is 10 years.)

Rather than accept the lab-worker data as definitive, Duesberg said he would like to see an epidemiologic study to answer the question of whether HIV causes AIDS. The study he wants would compare two large groups of people matched for age, lifestyle, and "non–drug use" who differ only in HIV status. "If the HIV-positive group had significantly more AIDS-defining diseases than the negative group, HIV could be the cause," Duesberg says. But, he says, "there is not even one study in the vast AIDS literature that shows that an HIV-positive group of 20- to 50-year-old people who do not use drugs and do not have congenital diseases, like hemophilia, have more AIDS diseases than an HIV-negative control group."



THE BETTMAN ARCHIVES

Postulator. Robert Koch.

Others contend that this study isn't necessary. "As far as I'm concerned, the laboratory workers prove causation," says Anthony Fauci, head of the National Institute of Allergy and Infectious Diseases. "I don't need any more than that."

–Jon Cohen

REVIEWING THE DATA–III

The Epidemic in Thailand

Two years ago, Peter Duesberg used epidemiological data from Thailand to argue that HIV doesn't cause AIDS. In his 1992 HIV/AIDS paper in *Pharmacology and Therapeutics*, Duesberg wrote: "An AIDS crisis that was reported to 'loom' in Thailand as of 1990 and that was predicted to 'explode' now has generated only 123 AIDS patients from 1984 until June 1991." But researchers familiar with the Thai epidemic argue that new data from Thailand present strong epidemiological arguments that HIV is indeed the cause of AIDS.

Thailand began extensive HIV-antibody testing in 1985, as documented by Bruce Weniger of the Centers for Disease Control and Prevention, with Thai co-workers, in a 1991 paper in the journal *AIDS*. By the end of 1987, nearly 200,000 HIV blood tests had been done on Thais from every known risk group—and there were fewer than 100 positive samples.

But then the virus began a rapid spread. By the end of 1988, in one risk group alone—users of injectable drugs—more than 1000 people had tested positive for HIV. Tim Brown, a theoretical physicist at the East-West Center in Hawaii who has worked with the Thai National Economic and Social Development board to model the AIDS epidemic, estimates that by the end of 1993 more than 700,000 Thais had become infected with HIV. "It's hard to think of any other country that has had such large amounts of spread that is well documented," says Weniger.

The linchpin of the argument that HIV causes AIDS in Thailand, say Weniger and others, is that the dramatic rise in HIV infections is being closely followed by a rise in AIDS cases. Brown's data show that, as of the end of 1993, there were more than 8000 cumulative AIDS cases. "The Thai data is quite compelling that HIV preceded AIDS, and the increase in HIV infection is now being seen in AIDS cases," says Weniger.

RAPID SPREAD

Year	Estimated HIV prevalence	New AIDS cases reported this year	Cumulative reported AIDS cases
1988	12,850	18*	18
1989	86,000	34	52
1990	297,000	91	143
1991	499,000	460	603
1992	634,000	1485	2088
1993	708,000	6026	8114

*Includes pre-1988 cases

SOURCE: TIM BROWN

Duesberg counters that there is "no explosion" of HIV infection in Thailand. "Instead, we look here at an explosion of HIV testing that began in 1989, and that has discovered a long-established, low incidence of HIV in Thailand," he wrote in reply to questions from *Science*. As for the rise in Thai AIDS cases, he agrees that there has been "a small explosion," which he writes "appears to be a consequence of new Thai sex and drug practices, not of a long-established latent retrovirus."

But Brown predicts that by 2000 there will be 1.4 million cumulative HIV infections and 480,000 AIDS cases in Thailand. "Thailand proves exactly why Duesberg is wrong," says Brown.

–Jon Cohen

Transmission of human immunodeficiency virus (HIV) in health-care settings worldwide

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Based on the information available, transmission of human immunodeficiency virus (HIV) can and does occur in health-care settings. No cases of such transmission have been reported from an infected health-care worker to a patient. Transmission of HIV from an infected patient to a health-care worker has been documented after parenteral or mucous-membrane exposure to blood. However, this risk is <1%, is limited to exposure to blood, and can be further minimized through adherence to routine infection control measures. Patient-to-patient transmission through invasive equipment or through HIV-infected blood, blood products, organs, tissues, or semen also occurs but can be prevented by proper sterilization of instruments and through donor-deferral, donor screening, and heat treatment of Factors VIII or IX to inactivate the HIV. In health-care settings, prevention of HIV transmission requires education of all health-care workers and ancillary staff, provision of necessary equipment, and strict adherence to general infection control practices.

Transmission of human immunodeficiency virus (HIV) in health-care settings can occur via instruments or equipment from the health worker to patient, from the patient to health worker, and from patient to patient. In September 1988, the Global Programme on AIDS of the World Health Organization initiated a project to bring together the available data from various countries on HIV transmission in each of these situations. Summarized below are the findings gathered from the WHO Collaborating Centres on AIDS.

Worker-to-patient transmission

Persons with HIV infection include health workers, many of whom continue to work in health-care settings. In the United Kingdom, a trainee surgeon who possibly contracted HIV infection occupationally in Africa, and who worked in urology, vascular surgery and general surgery, died of AIDS in 1988. Three hundred and thirty-six patients who had been operated upon by this surgeon were offered counselling and testing for HIV. Seventy-six (22%) chose to be tested and all were negative for HIV; 63 of these were tested over 90 days following exposure (Dr J. Porter, Public Health Laboratory Service, Communicable Disease Surveillance Centre, London, England; personal communication, 1989).

In an ongoing investigation in the USA, as of May 1989 none of an HIV-infected surgeon's 615 patients who were tested was positive for HIV antibody (Dr B. Mishu, Tennessee Department of Health and Environment, Nashville, USA; personal communication).

There are two retrospective published studies from the USA (*1*, *2*). The first looked for cases of AIDS among patients who had been treated by a surgeon with AIDS; no instances of HIV infection were identified. In the second report, HIV antibody testing was offered to patients who had been operated on by an HIV-infected surgeon. Of the 75 patients tested none was positive for HIV antibody.

No individual case reports of HIV transmission from an infected health-care worker to a patient have been published or reported.

Patient-to-worker transmission

Numerous studies of HIV transmission from patients to health workers are underway throughout the world (Table 1). Data are available from prospective studies in Canada, Italy, Spain, Sweden, United Kingdom and USA (*3–21*). For example in the USA, in a study by the Centers for Disease Control, of 1201 health workers with documented needlestick injuries, cuts with sharp objects, and contamination of open

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Table 1: **Prospective studies of health-care workers exposed to HIV-infected patients**[a]

Author and reference	No. of exposures	No. of workers	Number infected	Infection rate (per 100)
Marcus (*10*)	1201	1201	3	0.25
Gerberding (*15*)	625	212	1	0.47
Henderson (*11*)	482	359	1	0.27
Elmslie (*16*)	281	281	0	0.00
Pizzocolo (*20*)	195	195	0	0.00
Gill[b]	110	110	0	0.00
Hernandez (*18*)	58	58	0	0.00
Joline (*4*)	52	48	0	0.00
Ramsey (*19*)	44	44	1	2.27
Jorbeck (*21*)	41	41	0	0.00
Leach (*17*)	—	31	0	0.00

[a] Although these prospective studies are similar in following up health-care workers exposed to HIV-infected patients, their data may not be comparable because different eligibility criteria and definitions of exposure were used.
[b] Personal communication, 1989.

wounds or mucous membranes with the blood of HIV-infected patients, 4 were tested positive for HIV antibody. One of these workers was first tested positive 10 months after a needlestick injury, but non-occupational exposure to HIV infection could not be excluded. The other three persons had received needlestick injuries and were tested negative for HIV antibody at the time of the exposure, but experienced acute retroviral syndromes and then seroconverted to HIV. Two of these three needlesticks were caused by coworkers during resuscitation procedures. At the Clinical Center, National Institutes of Health, USA, 359 health-care workers with 482 parenteral or mucous-membrane exposures to the blood or other body fluids of HIV-infected patients were tested for HIV antibody; one, who was cut with a sharp object, experienced an acute retroviral syndrome and seroconverted (*11*). Also in the USA, at San Francisco General Hospital, 212 workers with 625 parenteral or mucous-membrane exposures to HIV-infected blood or body fluids were tested for HIV antibody; one of these with a needlestick injury experienced an acute retroviral syndrome and sero-converted to HIV (*15*). Of 44 health-care workers at the University of Texas Medical Branch, Galveston, Texas, USA, with percutaneous and non-percutaneous exposures to HIV-infected secretions, one with a needlestick injury seroconverted (*19*).

The other prospective studies have failed to document occupational transmission. In Canada, of 281 workers with parenteral or mucous-membrane exposure to HIV-infected patients enrolled in a national surveillance programme, none has seroconverted (K.D. Elmslie, Acting Director, Bureau of Epidemiology and Surveillance, Federal Centre for AIDS, Ottawa, Canada; personal communication, 1989). Of 110 health workers in the United Kingdom exposed to HIV infected blood via percutaneous injury, bite or contact with broken skin, or a splash, none has seroconverted (Dr N. Gill, UK Public Health Laboratory Service, Communicable Disease Surveillance Centre, London, England; personal communication, 1989). In Brescia, Italy, of 195 health-care workers with needlestick exposures to HIV-infected blood, none has seroconverted (*20*). In Sweden, surveillance of 41 health workers exposed to HIV-infected blood found no seroconversions (*21*). Other countries are conducting surveillance of health workers who are occupied with HIV-infected or AIDS patients at either a national or local level (e.g., France, Switzerland, Hungary, Greece). Data from these projects have not been systematically analysed. The combined data consistently demonstrate that the risk to health-care workers of occupationally acquiring HIV infection is less than 1% per needlestick exposure to HIV-infected blood.

Cross-sectional studies in Africa have shown that the prevalence of HIV-1 infection in health workers was not related to the degree of patient, blood, or needle contact and was considered to reflect the background rate of infection in the community (*22, 23*). In Kinshasa, Zaire, in 1984, 6.4% of 2384 health-care workers were tested positive for HIV antibody (*22*); the seropositivity rate increased to 8.7% in 1986 (*23*). In Dakar, Senegal, of 780 hospital workers, 1 (0.1%) was seropositive for HIV-1 and 4 (0.5%) were seropositive for HIV-2. The authors concluded that "hospital workers do not represent a risk group for HIV infection when compared with the healthy control population in Senegal" (*24*).

Additional studies have been conducted for

specific occupations, such as dentistry (*25, 26*) or for certain procedures, such as mouth-to-mouth resuscitation (*27*). Of 255 dental personnel with an estimated 110 potential contacts to probably infectious material, none was positive for HIV antibody (*25*). Klein and colleagues, in the USA, identified one seropositive dentist with no other identifiable risks for HIV infection among 1309 dental professionals tested for HIV antibodies (*26*). The HIV-infected dentist sustained numerous needlestick injuries and practised in an area of the USA with a high prevalence of AIDS cases. Four nurses attempted resuscitation of a patient with AIDS who committed suicide. The nurses were exposed to large amounts of the patient's blood by giving mouth-to-mouth resuscitation as the patient bled from the mouth and nose; 18 months after the incident the nurses remained negative for HIV antibody (*27*). In addition to these studies, individual cases of occupational transmission of HIV have been reported. The definition of occupational transmission of HIV varies with the reports and no standard definition has been published. The best documented reports of HIV transmission which can be attributed to occupational exposure are those in which the worker is tested and found negative for HIV antibody shortly after the exposure (preferably within 30 days), experiences an acute retroviral syndrome temporally associated with the exposure, and subsequently seroconverts, which generally occurs within 6 months after the exposure. The number of reports is small considering the number of health professionals who have cared for HIV-infected patients, regardless of whether the patient's HIV infection status was known. These case reports can be divided into cases for whom seroconversion was documented after a specific exposure (Table 2), and those presumptive or possible cases for whom serology or exposure data are lacking (Table 3). In this report, documented occupationally-related seroconversions are those that involved a specific exposure to HIV-infected blood, a baseline negative HIV antibody test in the worker followed by a positive test, regardless of the time interval between the two samples. In either documented or possible cases the health-care worker denies any other risk for HIV infection except occupational exposure.

Among the documented seroconversions, 13 reports were from the USA, two from France, and one each from the United Kingdom, Martinique, and Italy (*7, 10, 15, 28–38*) (Table 2). Of these 18 reports, 13 involved parenteral exposure (i.e., needlestick injury or cut with a sharp object) to blood or blood-containing body fluids, five were caused by blood contamination of mucous membranes or non-intact skin, and one was the result of parenteral exposure to concentrated HIV-1. An acute retroviral syndrome was reported in 14 of the 18 cases; in two cases details on an acute febrile illness are not proved. Six of these cases (*10, 15, 19, 29, 35*) were identified in various prospective studies discussed above.

Table 2: **Data from 18 documented seroconversions in health workers**

	Author and reference	Country	Type of exposure	ARS[a]
1.	Editorial (*28*)	United Kingdom	Needlestick	yes
2.	Stricof (*29*)	USA	Needlestick	yes
3.	Oksenhendler (*30*)	France	Needlestick	yes
4.	Neisson-Vernant (*31*)	Martinique	Needlestick	yes
5.	CDC[b] (*7*)	USA	Non-intact skin	yes
6.	CDC (*7*)	USA	Mucous membrane	no
7.	CDC (*7*)	USA	Non-intact skin	yes
8.	Gioannini (*32*)	Italy	Mucous membrane	yes
9.	Michelet (*33*)	France	Needlestick	yes
10.	Wallace (*34*)	USA	Needlestick	yes
11.	Barnes (*35*)	USA	Sharp object	yes
12.	Ramsey (*19*)	USA	Needlestick	no
13.	CDC (*9*)	USA	Needlestick	yes/AIDS
14.	Marcus (*10*)	USA	Needlestick	yes
15.	Marcus (*10*)	USA	Two needlesticks	yes
16.	Gerberding (*15*)	USA	Needlestick	yes
17.	Weiss (*37*), CDC (*38*)	USA	Sharp object	NR[c]
18.	CDC (*36*)	USA	Cutaneous	NR

[a] ARS = acute retroviral syndrome.
[b] CDC = Centers for Disease Control, USA.
[c] NR = not reported.

The cases that cannot be considered conversions, and may or may not represent occupational transmission, because of insufficient information, include six from the USA and one from the United Kingdom, Denmark, France, Mexico, Germany and Italy (*5, 6, 26, 37–46*) (Table 3). With one exception (*5, 6*), HIV antibody detection or the diagnosis of AIDS preceded identification of a potential occupational exposure to HIV.

Anecdotal reports of HIV infection in health-care workers, with or without documented seroconversion, emphasize the difficulty of determining whether infection was occupationally acquired. If substantial numbers of health-care workers were infected with HIV this would probably be reflected in national AIDS case reporting.

Although most countries do not report demographic or occupational information for AIDS cases, these data are available from the AIDS case surveillance dataset in the USA. While 5.4% of the AIDS cases reported working in a health-care setting since 1978, this is comparable to the proportion of

Table 3: **Data on 12 possible cases of occupational transmission of HIV**

	Author and reference	Country	Type of exposure
1.	Bygbjerg (*39*)	Denmark	Surgical practice in Zaire
2.	Belani (*40*)	USA	Palm prick from hospital waste
3.	Anonymous (*41*)	France	Worked in intensive care unit
4.	Grint (*42*)	United Kingdom	Home-health provider, non-intact skin
5.	Weiss (*5*), McCray (*6*)	USA	Colonic biopsy Needlestick
6.	Weiss (*5*), CDC (*43*)	USA	Two needlesticks
7.	Weiss (*5*), CDC (*43*)	USA	Two exposures/unknown source
8.	Weiss (*37*), CDC (*38*)	USA	Concentrated virus on skin
9.	Klein (*26*)	USA	Multiple needlesticks
10.	Ponce de Leon (*44*)	Mexico	Needlestick, puncture wound
11.	Schmidt (*45*)	Federal Republic of Germany	Needlestick
12.	Lima (*46*)	Italy	Needlestick

the total US population working in the health services (5.7%) (*47*); most (95%) of this group of AIDS patients have recognized non-occupational risk factors for HIV infection. The remaining cases with an unidentified risk are demographically more similar to other AIDS cases in the USA than they are to health-care workers (i.e., more likely to be male, nonwhite, and service rather than clinical workers) (*48–50*).

Patient-to-patient transmission

Transmission of HIV via contaminated needles and syringes or reused equipment in health-care settings has been reported from several countries. Syringes contaminated with blood aspirated during the course of intravenous injections were identified as the probable source of nosocomial HIV infection among 41 children hospitalized in the USSR (*51*). Medical injections were found to be a risk factor for HIV seropositivity among children under the age of 24 months in Kinshasa, Zaire, and among hospital workers (*52, 22*). However, other researchers in Rwanda did not find an association between the number of medical injections received and HIV seropositivity (*53*). These authors concluded that medical injections were given less frequently in Kigali, Rwanda, than in Kinshasa. Additional cases of transmission of HIV to blood or plasma donors in Spain and Mexico (*54–58*), to haemodialysis patients in Argentina (*59*), and after acupuncture in France (*60*) have also been reported. These cases emphasize the need for proper sterilization and disinfection of reusable equipment and increased use of disposable equipment when available.

HIV transmission through blood and blood products was documented early in the AIDS epidemic. Cases of HIV infection have also resulted from transplantation of infected organs, tissues, bone, and through semen used for artificial insemination (*61–63*). Donor self-deferral (persons excluding themselves from donating) and routine HIV-screening of donors of blood, blood products, organs, tissues, and semen have significantly reduced the risk of transmission from these sources. However, some areas of the world where routine HIV-screening is not yet available continue to have a serious problem with HIV transmission through blood and blood products.

Résumé

Transmission du virus de l'immunodéficience humaine dans les établissements de soins de santé

Des cas de transmission du virus de l'immunodéficience humaine (HIV) se sont effectivement produits dans des établissements de soins de santé, mais on ne connaît aucun cas où le virus ait été transmis à un patient par un agent de santé infecté. Des agents de santé ont été contaminés par le sang de patients infectés, soit à la suite de piqûres, soit par contact du sang avec les muqueuses. Toutefois, ce risque est inférieur à 1%, il n'existe que s'il y a contact avec le sang des malades, et il peut être encore réduit si les mesures habituelles de protection contre les infections sont rigoureusement respectées. La transmission de patient à patient par les instruments chirurgicaux ou par le sang, ses dérivés, les organes, les tissus ou le sperme infectés est possible, mais peut être évitée par diverses méthodes: stérilisation soignée des instruments, délais d'attente et mesures de dépistage imposés aux donneurs, traitement par la chaleur des facteurs VIII et IX pour inactiver le virus. La

prévention de la transmission du VIH dans les établissements de soins passe par l'éducation de tous les professionnels de la santé et du personnel auxiliaire, la mise en place de l'équipement nécessaire et le respect rigoureux des mesures générales de prévention des infections.

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SHORT REPORTS

HIV-1 and the aetiology of AIDS

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The belief that HIV-1 infection causes AIDS has been questioned, and the suggestion made that to know the correct cause of AIDS the incidence of disease in patients with and without risk behaviours and with and without antibody to HIV-1 must be known. We describe findings in such a cohort. In 715 homosexual men followed for a median of 8·6 years, all 136 AIDS cases occurred in the 365 individuals with pre-existing HIV-1 antibody. Most men negative for HIV-1 antibody reported risk behaviours but none developed any AIDS illnesses. CD4 counts fell in anti-HIV-1-positive men but remained stable in antibody-negative men, whether or not risk behaviours were present. The hypothesis that AIDS in homosexual men is caused not by HIV-1 infection but by drugs and sexual activity is rejected by these data. HIV-1 has an integral role in the pathogenesis of AIDS.

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Controversy continues to surround the question of whether human immunodeficiency virus 1 (HIV-1) infection causes the acquired immunodeficiency syndrome (AIDS).[1] The conventional hypothesis proposes that HIV-1 infection leads to depletion of CD4 cells and hence to progressive immune deficiency.[2] Some investigators have questioned this conventional "virus-AIDS" theory,[3] proposing instead a "risk-AIDS" hypothesis. The latter hypothesis asserts that it is not HIV-1 infection per se but rather the risk behaviours associated with HIV-1 infection that cause disease.[4,5] Duesberg has cited specifically chronic promiscuous male homosexual activity[5] and the use of drugs—including nitrite inhalants[6]—as exposure risks responsible for the epidemic of AIDS in homosexual men. He has stated that to identify the correct cause of AIDS the incidence of AIDS in controlled cohorts of risk-takers and non-risk-takers, with and without antibody to HIV-1, must be known, but that no such data are available.[7] We present the results of just such a controlled study.

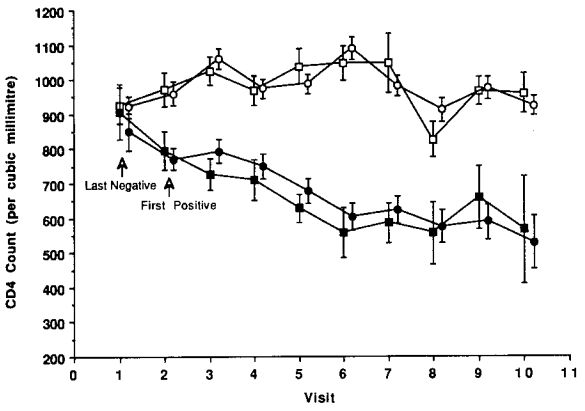
We have followed 715 homosexual men recruited from six general practices in central Vancouver.[8] During recruitment from November, 1982, to February, 1984, any homosexual patient already enrolled in the practice who attended for any reason was asked by his doctor to participate in the study. The refusal rate was 5%. Follow-up visits occurred about once every six months until September, 1986, after which time subjects attended annually. During each visit, subjects completed a self-administered questionnaire concerning lifestyle and illness, underwent a complete physical examination, and had blood samples drawn for immunologic and HIV-1 antibody testing.[8] Median duration of follow-up for all subjects was 8·6 years. As well as clinical follow-up, the British Columbia provincial and Canadian national AIDS registries were searched regularly from 1988 to identify any additional cases of AIDS in the entire cohort. Seroprevalent men (n = 237, 33%) were defined as those who were HIV-1-antibody positive when they entered the study, seroincident men (n = 128, 18%) as those who seroconverted while under study, and seronegative men (n = 350, 49%) as those who remained HIV-1-antibody negative at the most recent follow-up visit. The seroprevalent and seroincident groups combined are referred to as the seropositive group. All reported p values are two-sided.

By April, 1992, 136 cases of AIDS-related illnesses had been diagnosed in our cohort. These illnesses included *Pneumocystis carinii* pneumonia (54), Kaposi's sarcoma (34), lymphoma (11), oesophageal candidiasis (8), *Mycobacterium avium-intracellulare* infection (7), cytomegalovirus infection (4), wasting syndrome (4), and other illnesses (14). Every case of AIDS-associated illness occurred in individuals with pre-existing HIV-1 antibody, and no AIDS illnesses occurred in men who remained persistently negative for HIV-1 antibody.* There were 101 AIDS-related deaths in the seropositive men. Excluding AIDS-related mortality, there were 6 deaths in the seropositive men: 2 cases of hepatitis, 1 lung cancer, 1 homicide, 1 suicide, and 1 drug overdose. In the seronegative group, there were only 2 deaths—1 myocardial infarction and 1 suicide—and no deaths due to any AIDS-related conditions.

To account for risk behaviours in our subjects, we undertook analyses involving use of nitrite inhalants (ever versus never) and illicit drugs (ever used cocaine, heroin, amphetamines, lysergic acid diethyl amide, or methylene-dioxy amphetamine), and increased frequency of receptive anal intercourse (≥25% of sexual encounters; this cut-off was chosen a priori to achieve an approximately equal division). Lifetime prevalences of these behaviours were similar in the 136 seropositive men who developed AIDS and in the 229 seropositive men who did not develop AIDS: use of nitrite inhalants, 88% in both groups; use of illicit drugs, 75% and 80%, respectively; and increased frequency of receptive anal intercourse, 78% and 82%, respectively. Since these risk behaviours are known to correlate to HIV-1 infection, it is not surprising that lifetime prevalences in the 350 seronegative men were lower than in the seropositive group—ie, 56%, 74%, and 58% for nitrite inhalants, illicit drugs, and increased frequency of receptive anal intercourse, respectively. However, risk behaviours were present at appreciable levels in the seronegative group without development of a single case of AIDS.

To corroborate absence of AIDS and associated effects in the seronegative group, we studied CD4 count as a measure of immune impairment. The seronegative group was compared with the seroincident group to allow observation of the entire natural history of HIV-1 infection

*A table showing AIDS-related illnesses in the three patients groups, a figure showing changes in CD4 count over ten follow-up visits in seroincident and seronegative patients, and figures showing changes in CD4 counts in seroincident and seronegative patients stratified for use of illicit drugs and for frequency of receptive anal intercourse are available on request from *The Lancet*.



CD4 counts for seronegative and seroincident groups stratified by use of nitrite inhalants.

For the seronegative group, all visits are used, whereas for the seroincident group visits begin with the last anti-HIV-1-negative and first anti-HIV-1-positive results (arrows). Seronegative men who ever used (○) and never used (□) nitrite inhalants; seroincident men who ever used (●) and never used (■) nitrite inhalants. Points are mean (SE).

with control for the duration of infection. For the seroincident group, baseline was the time of the last seronegative test result and the final CD4 count was obtained from the ninth visit after seroconversion. The seroincident and seronegative groups were similar at baseline. The average rate of CD4 decline in the seroincident group, based on linear regression, was about 50 cells/μL per year (95% CI 39–61 cells/μL per year; $p = 0.0001$ for difference from zero), and the final mean CD4 count in the seroincident group was 547 cells/μL ($n = 25$; 414–680). By contrast, the average rate of CD4 decline in the seronegative group was not significantly different from zero ($p = 0.73$), with mean baseline and final CD4 counts of 921 (868–974) cells/μL and 937 (886–988) cells/μL, respectively.*

Stability of CD4 counts in anti-HIV-1-negative men and decline in counts in seroincident men were apparent whether or not nitrite inhalants were used (figure). A multiple regression model was fitted to explain final CD4 count in terms of the baseline CD4 count, serological group (seronegative or seroincident), and the use of nitrite inhalants. Baseline CD4 count and serological group were highly significant in explaining final CD4 count ($p < 0.0001$ in each case), but inhalant use was not significant ($p = 0.21$). Similar analyses of CD4 counts were done after stratification for use of illicit drugs and for frequency of receptive anal intercourse. In both cases, stable counts were found in the seronegative group and declining counts in the seroincident group, regardless of the presence or absence of the HIV-1 risk behaviour,* and multiple regression models showed both baseline CD4 count and the serological group to be highly significant in explaining the final CD4 count, but that risk behaviour was not significant.

The risk-AIDS hypothesis[2-6] that AIDS in homosexual men is caused not by HIV-1 infection but by other exposures, such as drug use and male homosexual activity, is clearly rejected by our data. The evidence supports the hypothesis that HIV-1 has an integral role in the CD4 depletion and progressive immune dysfunction that characterise AIDS. A central role for HIV-1 in the pathogenesis of AIDS does not rule out a role for cofactors that might help to determine the clinical course in different hosts. Whether these cofactors involve other microorganisms, genetic susceptibility, autoimmune processes, or other phenomena is currently the subject of debate and investigation. However, it is a disservice to the many people infected with HIV-1 and a hindrance to public health initiatives for scientists to claim that HIV-1 is harmless and not aetiologically related to AIDS.

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Magnetic resonance neurography

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Radiological methods exist for generating tissue-specific images of bone, vessels, lymphatics, abdominal viscera, and the central nervous system, but there has been no reliable means to generate a clinical image of a nerve. We present the first "image neurogram" and report a method for producing such images by use of commercial magnetic resonance imaging systems. The image depicts a human nerve in situ in relation with a nerve graft, wherein the nerve is rendered in isolation much like a vessel appears in isolation in a subtraction angiogram.

Lancet 1993; **341**: 659–61.

Pain and loss of function due to neuropathy, nerve compression, and traumatic nerve injury prompt many millions of diagnostic tests every year. However, the surgical treatment of suspected nerve compression is often hindered, and is sometimes precluded, because the site of compression cannot be accurately localised. The cranial, peripheral, and autonomic nerves have remained the preserve of physical and electrical diagnosis because no imaging technique has so far proved adequate. Useful cross-sectional images of nerves cannot be made by computerised tomographic scanning.

Birth Defects Among Children Born to Human Immunodeficiency Virus-Infected Women

Pediatric AIDS Clinical Trials Protocols 219 and 219C

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Background: Some studies have detected associations between in utero antiretroviral therapy (ARV) exposure and birth defects but evidence is inconclusive.

Methods: A total of 2202 human immunodeficiency virus (HIV)-exposed children enrolled in the Pediatric AIDS Clinical Trials Group 219 and 219 C protocols before 1 year of age were included. Birth defects were classified using the Metropolitan Atlanta Congenital Defects Program coding. Logistic regression models were used to evaluate associations between first trimester in utero ARV exposure and birth defects.

Results: A total of 117 live-born children had birth defects for a prevalence of 5.3% (95% confidence interval [CI]: 4.4, 6.3). Prevalence did not differ by HIV infection status or overall ARV exposure; rates were 4.8% (95% CI: 3.7, 6.1) and 5.8% (95% CI: 4.2, 7.8) in children without and with first trimester ARV exposure, respectively. The defect rate was higher among children with first trimester efavirenz exposure (5/32, 15.6%) versus children without first trimester efavirenz exposure (adjusted odds ratio [aOR] = 4.31 [95% CI: 1.56, 11.86]). Protective effects of first trimester zidovudine exposure on musculoskeletal defects were detected (aOR = 0.24 [95% CI: 0.08, 0.69]), while a higher risk of heart defects was found (aOR = 2.04 [95% CI: 1.03, 4.05]).

Conclusions: The prevalence of birth defects was higher in this cohort of HIV-exposed children than in other pediatric cohorts. There was no association with overall ARV exposure, but there were some associations with specific agents, including efavirenz. Additional studies are needed to rule out confounding and to evaluate newer ARV agents.

Key Words: in utero exposure, antiretroviral therapy, congenital abnormalities/anomalies, HIV

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Since 1998, the US Public Health Service has recommended the use of combination antiretroviral therapy (ARV) to prevent mother-to-child human immunodeficiency virus (HIV) transmission.[1] Because zidovudine and other nucleoside analogues can affect nuclear and mitochondrial deoxyribonucleic acid replication, the safety of in utero exposure to these drugs is of concern.[2] In addition, there is inadequate fetal and neonatal safety data for non-nucleoside analogues and protease inhibitors. Efavirenz, a non-nucleoside analogue, is considered a potential teratogen on the basis of animal data and case reports.[1,3–6]

While existing data on in utero ARV exposure and birth defects have been mostly reassuring,[7–9] some studies have reported elevated risks with specific exposures[10,11]; others have been limited by small sample size or possible confounding. The US Woman and Infants Transmission Study documented a birth defect rate of 3.56 per 100 live births in 2527 infants born to HIV-infected women from 1990 through 2000,[12] which was not significantly different than the rate major of defects of 2.76 per 100 live births in the general pediatric population estimated by the Metropolitan Atlanta Congenital Defects Program (MACDP).[11] However, first trimester zidovudine exposure was significantly associated with an increased risk of hypospadias among male infants. The US Antiretroviral Pregnancy Registry (APR) estimated an overall prevalence of defects of 2.9% (95% confidence interval [CI]: 2.4, 3.5) among greater than 4300 first trimester ARV exposed children, which did not differ from the rate among children exposed in later trimesters.[13] The Pediatric AIDS Clinical Trials Group (PACTG) protocols 219 and 219C provided an opportunity to further estimate the independent association between in utero ARV exposure, including newer agents, and birth defects.

METHODS

Study Population

The source population was children enrolled in PACTG protocols 219 and 219C, a multisite US cohort of children born to HIV-infected women initiated to study the long-term effects of in

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utero ARV exposure and complications of pediatric HIV infection.[14] Protocol 219 followed HIV-infected and HIV-uninfected perinatally exposed children at clinics across the United States from May 1993 through August 2000. Children currently or previously enrolled in another PACTG protocol and children whose mothers were enrolled in a PACTG perinatal protocol during pregnancy were eligible. In September 2000, a revised protocol was initiated, PACTG 219C, and the eligibility criterion mandating enrollment in another PACTG protocol was removed. The present study was restricted to children enrolled in 219 or 219C before 1 year of age to improve the accuracy of birth defect information recorded on protocol case report forms. The study was approved by site institutional review boards, and parents or guardians provided informed consent.

Data Collection

Study visits, which included physical examinations, were scheduled every 3 months for HIV-infected children, and every 6 months until 2 years of age (protocol 219), or every 3 months through 1 year of age (protocol 219C) and annually thereafter for HIV-uninfected children. Protocol 219 did not include a direct question regarding the presence of defects, but birth defects were a primary outcome and were recorded on diagnosis case report forms. Protocol 219C included a direct question regarding birth defects. Detailed data on birth defects also were collected in PACTG perinatal protocols 076, 185, 249, 250, 316, 332, 353, 354, 358, and 386 and the International Maternal Pediatric and Adolescent AIDS Clinical Trials (IMPAACT) protocol P1025. Forty-two percent of mother-infant pairs in protocol 219 and 219C participated in one of these perinatal protocols during pregnancy-gestation; these data were used to supplement 219 and 219C data.

Exposure

Gestational age at birth was estimated from the date of last menstrual period, ultrasound measurement before 22 weeks gestation, or newborn examination. Trimesters were defined as first trimester, conception to <14 weeks gestation; second trimester, 14 weeks to <28 weeks gestation; and third trimester, 28 weeks to delivery. The primary determinant was first trimester in utero ARV exposure. We considered overall ARV exposure, ARV classes, and specific ARV agents to which at least 1 child with a birth defect was exposed in the first trimester. The reference group consisted of children unexposed to the particular ARV drug (or class) during the first trimester, and thus included ARV unexposed children, children exposed to ARV in labor only, children unexposed to the particular ARV drug but to other ARV, and children exposed to the particular ARV drug in the second and/or third trimester only.[15] We also examined ARV exposure by trimester of first exposure (unexposed, first trimester, second or third trimester); however, since the first trimester estimates were substantially unchanged in this model from the former classification, results from the more parsimonious models were presented.

Outcome

The outcome was the presence of a birth defect documented within the first year of life. Clinicians blinded to ARV exposure reviewed and classified the reported defects according to the MACDP guidelines as major defects or conditional defects.[16] To further prevent misclassification, we followed a modified version of MACDP guidelines employed by the APR,[13] in which children with 2 or more conditional defects in the absence of a major defect were considered a case. Therefore, a child with at least 1 major defect or at least 2 conditional defects in the absence of a major defect was considered a case. Children classified as having birth defects solely based on conditional MACDP defects were categorized separately from those with major defects.

Statistical Analysis

The prevalence and exact 95% CI of birth defects per 100 live births was estimated overall, by cohort (219 vs. 219C), and infant HIV-infection status. Differences in birth defect prevalence across these and other characteristics were assessed using the χ^2 test, Fisher exact test, and Cochran-Armitage trend test for categorical variables, and the Wilcoxon rank sum test for continuous variables. Logistic regression models were used to estimate associations between first trimester in utero ARV exposure of any drug and of specific drugs and the most common categories of birth defects (all birth defects, musculoskeletal defects, and heart defects), including both HIV-infected and uninfected children. Potential confounders with a $P < 0.25$ in univariate analysis were initially included in adjusted models, but only those that produced at least a 10% change in the estimated odds ratio were retained in final models. Children with recognized chromosomal abnormalities or congenital infections such as toxoplasmosis were excluded from regression analyses.

RESULTS

Of 5931 children in protocols 219 and 219C, 2202 enrolled by 1 year of age and constituted the study population. Following clinical review of birth defects according to MACDP guidelines, 117 children had at least 1 defect, 103 with at least 1 major defect, and 14 with 2 or more conditional defects but no major defect. Among these 117 children, 77 had 1 birth defect, 30 had 2 birth defects, 6 had 3 birth defects, and 4 had 4 birth defects. Overall defect prevalence was 5.3% (95% CI: 4.4, 6.3) including all 117 cases, and was 4.7% (95% CI: 3.8, 5.6) including 103 cases with major defects. Prevalence was 4.9% (95% CI: 2.6, 8.2) and 5.4% (95% CI: 4.4, 6.5) in HIV-infected and HIV-uninfected/indeterminate children (Table 1), respectively, and was 4.8% (95% CI: 3.7, 6.1) in first trimester unexposed children, and 5.8% (95% CI: 4.2, 7.8) in first trimester ARV exposed children (Table 2).

The majority of defects occurred in the heart and musculoskeletal system (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A514). Prevalence was significantly higher among children whose mother had participated in a PACTG study during pregnancy and increased with increasing maternal age (Table 1). Prevalence also was higher among males and children with first trimester folate antagonist exposure (ie, trimethoprim/sulfamethoxazole), although these differences were not statistically significant, and folate antagonist exposure was unavailable for over half of the children. There was no difference in defect prevalence by highest $\log_{10}$ median maternal HIV viral load (3.4 copies/mL [children with defects] vs. 3.5 copies/mL [children without defects]), or lowest median maternal CD4 count (360 cell/mL [children with defects] vs. 372 cells/mL [children without defects]) during pregnancy. Defect prevalence significantly differed by protocol: rates were 6.8% (95% CI: 5.2, 8.7) and 4.4 (95% CI: 3.3, 5.6) for children enrolled in protocol 219 (whether or not in 219C) and in 219C alone. Figure, Supplemental Digital Content 2, http://links.lww.com/INF/A513, shows the prevalence of birth defects by year of birth; 1992 and 2006 were excluded because of the small number of children born in these years. No overall difference in prevalence by year of birth was identified.

The unadjusted and adjusted estimates between first trimester in utero ARV exposure and birth defects are shown in Table 2. In unadjusted analyses, there was no significant association with overall first trimester ARV exposure or first trimester exposure to specific drug classes. However, significantly more children with birth defects were exposed to efavirenz in the first trimester. The

TABLE 1. Prevalence of at Least One Major or at Least 2 Conditional Birth Defects by Infant Characteristic of Children in PACTG Protocols 219 and 219C

Characteristic	Birth Defect (N = 117)		No Defect (N = 2085)		P*
	N	%	N	%	
HIV infection status					
Infected	13	4.9	254	95.1	0.58
Uninfected	104	5.4	1814	94.6	
Indeterminate	0	0	17	100	
Sex					
Female	50	4.5	1061	95.5	0.09
Male	67	6.1	1024	93.9	
Race/ethnicity					
Non-Hispanic white	17	7.5	209	92.5	0.30
Non-Hispanic black	58	4.6	1199	95.4	
Hispanic	38	5.7	633	94.3	
Other	1	4.0	24	96.0	
Unknown	3	13.0	20	87.0	
Year of birth					
1992–1996	26	5.4	454	94.6	0.24
1997–2001	54	6.2	820	93.8	
2002–2006	37	4.4	811	95.6	
Protocol					
219 ± 219C	58	6.8	794	93.2	0.013
219C only	59	4.4	1291	95.6	
Earliest year of enrollment in 219 or 219C					
1993–1996	23	5.5	394	94.5	0.037
1997–2000	40	7.3	507	92.7	
2001–2006	54	4.4	1184	95.6	
Enrolled in perinatal study during gestation					
Yes	76	8.3	838	91.7	<0.0001
No	41	3.2	1247	96.8	
Maternal age at birth (yr)					
<20	5	3.5	138	96.5	0.049
20–<25	23	4.6	474	95.4	
25–<30	32	5.7	534	94.4	
30–<35	27	5.4	472	94.6	
≥35	21	7.1	274	92.9	
Unknown	9	4.5	193	95.5	
Gestational age at birth (wk)					
<32	6	12.0	44	88.0	0.33
32–<37	18	6.3	268	93.7	
≥37	65	6.8	892	93.2	
Unknown	28	3.1	881	96.9	
Birth weight (g)					
<2500	28	7.0	370	93.0	0.10
≥2500	89	5.0	1706	95.0	
Unknown	0	0	9	100	
First trimester in utero folate antagonist exposure					
Unexposed	68	8.0	785	92.0	0.08
Exposed	7	16.3	36	83.7	
Unknown	42	3.2	1264	96.8	
In utero alcohol exposure					
Unexposed	41	5.3	732	94.7	0.25
Exposed	16	7.4	201	92.6	
Unknown	60	5.0	1152	95.0	
In utero tobacco exposure					
Unexposed	35	5.3	621	94.7	0.44
Exposed	20	6.6	284	93.4	
Unknown	62	5.0	1180	95.0	
In utero marijuana exposure					
Unexposed	49	6.1	760	93.9	0.18
Exposed	5	3.3	145	96.7	
Unknown	63	5.1	1180	94.9	
In utero cocaine exposure					
Unexposed	47	5.9	754	94.1	0.38
Exposed	8	4.2	181	95.8	
Unknown	62	5.1	1150	94.9	
In utero heroin exposure					
Unexposed	51	5.6	852	94.4	1.00
Exposed	3	5.0	57	95.0	
Unknown	63	5.1	1176	94.9	
In utero methadone exposure					
Unexposed	54	5.7	890	94.3	0.43
Exposed	4	8.5	43	91.5	
Unknown	59	4.9	1152	95.1	

*P value from χ^2 test, Fisher exact test (in utero heroin exposure), or Cohrane-Armitage trend test (maternal age); subjects with unknown data excluded.

PACTG indicates Pediatric AIDS Clinical Trials Group.

mothers of all 5 cases were taking efavirenz at the time of conception and 3 stopped efavirenz around the time pregnancy would have been identified; the other 2 mothers stopped efavirenz in the second trimester. All mothers of the 5 efavirenz-exposed children with defects also were receiving lamivudine plus other ARV. The defects of these efavirenz exposed children included laryngomalacia (N = 1), meningomyelocele with Arnold-Chiari Malformation Type II (N = 1), hypospadias (N = 1), varus feet and hypertonicity of extremities (N = 1), and cleft palate (N = 1).

The rate of birth defects also was higher in children exposed to lopinavir/ritonavir in the first trimester than in children unexposed to lopinavir/ritonavir in the first trimester. The defects of the 6 lopinavir/ritonavir exposed children included hydronephrosis (N = 1), supernumerary nipple and umbilical hernia (N = 1), atrial septal defect (N = 1), pyloric stenosis (N = 2), and ventricular septal defect and hemangioma (N = 1). None of the children with defects were exposed to both efavirenz and lopinavir/ritonavir in the first trimester.

In models adjusted for first trimester folate antagonist exposure, year of birth, and perinatal study participation, the association with efavirenz persisted while the association with lopinavir/ritonavir was marginally significant ($P = 0.07$). To further explore possible confounding, we examined maternal and infant characteristics by perinatal protocol participation (data not shown). In models adjusted for year of birth, participation in a perinatal protocol was higher among infants with first trimester exposure to any ARV (Odd ratio [OR] = 1.47, 95% CI: 1.21, 1.79) and to any nucleoside analogue (OR = 1.48, 95% CI: 1.22, 1.80), and was lower among infants with first trimester exposure to any non-nucleoside analogue (OR = 0.57, 95% CI:0.38, 0.85). However, other characteristics generally were in the direction of a higher possible risk of defects in those who did not participate in a perinatal protocol (eg, more mothers <20 and >30 years of age, more maternal cocaine use, lower infant birth weights, more preterm births, and more HIV-infected infants) except for maternal alcohol use, which was higher among perinatal study participants.

We also examined associations between in utero ARV exposure and the most common categories of specific defects: musculoskeletal and heart (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A514). Because of the lower number of cases (N = 36 and 34, respectively),[2] these models were only adjusted for perinatal protocol participation and first trimester folate antagonist exposure. Protective effects of first trimester zidovudine exposure on musculoskeletal defects were detected in unadjusted (OR = 0.30, 95% CI: 0.10, 0.84) and adjusted models (OR = 0.24, 95% CI: 0.08, 0.69). Protective effects on musculoskeletal defects also were found with overall first

TABLE 2. Prevalence and Odds Ratio of at Least 1 Major or at Least 2 Conditional Birth Defects According to First Trimester in Utero ARV Exposure Among Children in Protocols 219 and 219C*

First Trimester in Utero Exposure	Birth Defect (N = 105)		No Defect (N = 1928)		Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
	N	%	N	%		
Any antiretroviral						
Unexposed‡	61	4.8	1209	95.2	Ref.	Ref.
Exposed	44	5.8	719	94.2	1.21 (0.81, 1.81)	1.10 (0.72, 1.67)
Nucleoside/nucleotide analogues						
Unexposed	61	4.8	1218	95.2	Ref.	Ref.
Exposed	44	5.8	710	94.2	1.24 (0.83, 1.84)	1.12 (0.73, 1.69)
Abacavir						
Unexposed	100	5.1	1854	94.9	Ref.	Ref.
Exposed	5	6.3	74	93.7	1.25 (0.50, 3.17)	1.50 (0.57, 3.96)
Didanosine						
Unexposed	104	5.2	1882	94.8	Ref.	Ref.
Exposed	1	2.1	46	97.9	0.39 (0.05, 2.88)	0.34 (0.05, 2.57)
Lamivudine						
Unexposed	69	4.7	1394	95.3	Ref.	Ref.
Exposed	36	6.3	534	93.7	1.36 (0.90, 2.06)	1.37 (0.87, 2.16)
Stavudine						
Unexposed	95	5	1814	95	Ref.	Ref.
Exposed	10	8.1	114	91.9	1.68 (0.85, 3.30)	1.53 (0.76, 3.09)
Tenofovir						
Unexposed	101	5.1	1887	94.9	Ref.	Ref.
Exposed	4	8.9	41	91.1	1.82 (0.64, 5.19)	1.39 (0.45, 4.34)
Zidovudine						
Unexposed	72	5	1356	95	Ref.	Ref.
Exposed	33	5.5	572	94.5	1.09 (0.71, 1.66)	0.98 (0.64, 1.52)
Non nucleoside analogues						
Unexposed	97	5.1	1794	94.9	Ref.	Ref.
Exposed	8	5.6	134	94.4	1.10 (0.53, 2.32)	1.46 (0.67, 3.16)
Efavirenz						
Unexposed	100	5	1901	95	Ref.	Ref.
Exposed	5	15.6	27	84.4	3.52 (1.33, 9.34)	4.31 (1.56, 11.86)
Nevirapine						
Unexposed	100	5.2	1815	94.8	Ref.	Ref.
Exposed	5	4.2	113	95.8	0.80 (0.32, 2.01)	1.05 (0.41, 2.70)
Protease inhibitors						
Unexposed	82	4.9	1598	95.1	Ref.	Ref.
Exposed	23	6.5	330	93.5	1.36 (0.84, 2.19)	1.36 (0.81, 2.28)
Indinavir						
Unexposed	101	5.1	1879	94.9	Ref.	Ref.
Exposed	4	7.5	49	92.5	1.52 (0.54, 4.29)	1.50 (0.51, 4.35)
Lopinavir/ritonavir						
Unexposed	99	5	1886	95	Ref.	Ref.
Exposed	6	12.5	42	87.5	2.72 (1.13, 6.55)	2.46 (0.93, 6.52)
Nelfinavir						
Unexposed	92	5.1	1719	94.9	Ref.	Ref.
Exposed	13	5.9	209	94.1	1.16 (0.64, 2.11)	1.23 (0.66, 2.30)
Saquinavir						
Unexposed	104	5.2	1899	94.8	Ref.	Ref.
Exposed	1	3.3	29	96.7	0.63 (0.09, 4.67)	0.46 (0.06, 3.49)

*Four children with trisomy 21 and 1 child with congenital toxoplasmosis excluded; 7 and 157 children with and without birth defects excluded due to unknown timing of in utero antiretroviral exposure.

†Adjusted for participation in a PACTG perinatal study, first trimester folate antagonist exposure and year of birth.

‡Includes children unexposed to any ARV during gestation (14 children with defects and 272 children without defects) and children exposed to ARV in the second and/or third trimester only.

ARV indicates antiretroviral therapy; OR, Odds Ratio; 95% CI, 95% confidence interval; PACTG, Pediatric AIDS Clinical Trials Group.

trimester ARV exposure and any first trimester nucleoside analogue exposure in adjusted models. These latter findings appeared to be driven by zidovudine exposure; the frequency of exposure was similar for any ARV, for any nucleoside analogue, and for zidovudine. In contrast, significantly more children with heart defects—MACDP category of heart, other, which excludes conotruncal and obstructive defects (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A514) were exposed to zidovudine in the first trimester in unadjusted (OR = 2.11, 95% CI: 1.07, 4.16) and adjusted models (OR = 2.04, 95% CI: 1.03, 4.05). This association was marginally significant when conotruncal and obstructive defects were included (OR = 1.78, 95% CI: 0.93, 3.40, $P = 0.08$).

To examine possible selection bias, we assessed enrollment into 219 and 219C of children who participated in PACTG 076, 316 or IMPAACT P1025 by defect status and in utero ARV exposure. These latter 3 studies were examined because birth defect information was collected and reviewed in these studies by the 076, 316, and P1025 investigators. It should be noted that 74%

of children in 219 and 219C who participated in a perinatal protocol were in one of these studies. Among children who participated in PACTG 076, 316 or IMPAACT P1025, more children with defects (31.2%) than without defects (24.8%) enrolled in protocols 219 and 219C ($P = 0.054$). However, the only important differences in enrollment by defect status and in utero ARV exposure were among children without defects: enrollment was higher among children unexposed to abacavir (17.0% exposed vs. 25.2% unexposed enrolled, $P = 0.048$), and exposed to saquinavir (44.4% exposed vs. 24.6% unexposed enrolled, $P = 0.018$). This differential enrollment among children without defects would increase and decrease estimated associations with abacavir and saquinavir exposure, respectively. No other evidence of selection bias was identified.

DISCUSSION

In HIV-uninfected and HIV-infected children enrolled in protocols 219 and 219C by 1 year of age, we documented a birth defect prevalence of 5.3% including all 117 cases, and 4.7% including 103 major cases only. No differences were found according to infant HIV infection status. While we did not detect an association between overall in utero ARV exposure and defects, associations with particular ARV drugs were identified.

Our study is the first to provide evidence of an association between efavirenz and birth defects in a population-based investigation, although the small number of infants with first trimester efavirenz exposure must be considered. Of the 5 children in our study with birth defects and first trimester efavirenz exposure, only 1 had a neural tube defect and has previously been described[5] and retrospectively reported to the APR. In prospectively reported APR cases, defects were detected in 13 (3.2%) of 407 live births with first trimester efavirenz exposure, which was similar to the overall APR rate; no specific pattern of defects was observed (1 case of meningomyelocele and 1 case of facial cleft with anophthalmia).[13] However, 3 (15%) of 20 infant cynomolgus monkeys with first trimester efavirenz exposure at levels similar to human exposure had defects (anencephaly and unilateral anophthalmia, micro-ophthalmia, and cleft palate).[6] We also detected associations between first trimester lopinavir/ritonavir exposure and defects, but this did not remain significant after adjustment for other covariates, perhaps because of low power. Animal studies have not demonstrated teratogenic effects, but have shown delayed skeletal ossification and skeletal variation at maternally toxic doses.[1]

The rate of birth defects in our cohort was higher than the 2.9% prevalence reported by the APR.[13] Other US[12] and European[8] studies of children born to HIV-infected women have not reported an elevated defect prevalence of birth defects, excluding the PACTG 076 randomized trial in which a rate of major defects of 8% was detected, and all ARV exposure occurred after the first trimester.[17] It is possible that differential ascertainment across studies could account for the differences. A total of 636 children in our study population had echocardiograms, most per study protocol, and more children with (41%) than without defects (28%) had echocardiograms. Early screening echocardiography can detect important subclinical malformations and produce rates of cardiac defects of 5% to 10% higher than expected.[18,19] Additionally, children whose mother had participated in a perinatal protocol were more likely to have a birth defect, possibly suggesting differential ascertainment.

To investigate potential selection bias, we examined enrollment into 219 and 219C among children who had participated in perinatal protocols PACTG 076, 316 and IMPAACT P1025. Despite the higher enrollment of children with defects into our cohort, it was nondifferential with respect to most in utero ARV exposures, and importantly, those with which we detected notable associations. Selection bias of our estimated associations between defects and ARV exposure is not of major concern. It should also be noted that IMPAACT P1025 is a cohort study and no ARV was given as part of the protocol[20]; likewise, in PACTG 316, all women were on clinically indicated ARV and the only randomized component was single-dose nevirapine at labor and delivery.[21]

To control for possible confounding, models were adjusted for perinatal protocol participation, exposure to folate antagonists, and year of birth. We examined other potential confounders of the association between in utero ARV exposure and birth defects, including maternal drug use, but had incomplete information. Some residual confounding may persist. Finally, because of the large number of ARVs available for use during pregnancy, it is impossible to adjust for all other ARVs when estimating effects of a particular ARV, and this should be considered in weighing the evidence from our study as well as other studies.

It is possible that some associations might have been attenuated if particular defects result from exposure to a particular ARV. We attempted to look at more refined categories of birth defects where power was sufficient. A lower risk of musculoskeletal defects and a higher risk of heart defects were found with first trimester zidovudine exposure. These findings were based on a small number of cases and require confirmation in other studies. An association between first trimester zidovudine exposure and septal heart defects was noted in PACTG protocol 185 and in a German study, although selection bias could not be ruled out.[13]

A potential limitation of our study is that children, not pregnant women, enrolled in protocols 219 and 219C. Therefore, birth defects resulting in fetal loss were not included. Birth defects in stillbirths occurring after 20 weeks gestation were included in the Women and Infants Transmission Study[12] and the APR.[13] If defects caused by a specific exposure resulted in an increase in stillbirths then our estimates would likely be attenuated.

In this US cohort of children born to HIV-infected women, we identified a higher prevalence of birth defects than other studies. Overall, first trimester in utero ARV exposure was not associated with an increased risk of defects. However, some associations with first trimester in utero exposure to particular ARVs were identified. Further study is needed to rule out possible confounding, and to examine associations between ARV exposure and specific birth defects. Practitioners are urged to report all pregnant women receiving ARV during pregnancy to the APR (www.APRegistry.com) as early as possible and preferably before the pregnancy outcome is known.

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Current Abstracts

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Multistate Outbreaks of Human *Salmonella typhimurium* Infections Associated With Pet Turtle Exposure—United States, 2008

Centers for Disease Control and Prevention. *MMWR*. 2010;59:191–196.

In September 2008, the Philadelphia Department of Public Health and the Pennsylvania Department of Health notified Centers for Disease Control and Prevention of an outbreak of possible turtle-associated human *Salmonella typhimurium* infections detected by identifying strains with similar pulsed-field gel electrophoresis patterns in PulseNet, a national molecular subtyping network for foodborne disease surveillance. The results of that investigation are summarized in this report.

A total of 135 cases in 25 states and the District of Columbia were identified in the national PulseNet database. Among 124 patients for whom demographic information was available, median age was 7 years (range: <1–94 years), and 54 (45%) patients were aged 5 years or younger; 63 (51%) were female. Seventy-eight percent of illnesses occurred during June to September.

Of 83 patients interviewed using a more extensive questionnaire, 35 (42%) had bloody diarrhea and 29 (35%) were hospitalized. No deaths were reported. Twenty (24%) of the 83 patients attended day care. Of 70 patients with primary cases, 26 (37%) reported exposure to turtles and 21 reported exposure to small turtles.

Among the 69% of patients who knew the source of the turtle, the majority of turtles were purchased from street vendors, flea markets, and nonpet stores (eg, souvenir or gift shops). Seven (10%) of the 70 primary patients reported other reptile exposures (eg, snakes or iguanas).

The Federal government prohibited sales of turtles with shell lengths <4 inches in 1975, after investigations demonstrated that small turtles were a major source of human *Salmonella* infections, particularly in children. Implementation of the prohibition resulted in a substantial decline in turtle-associated human salmonellosis, preventing an estimated 100,000 *Salmonella* infections annually in US children. Turtle-associated human salmonellosis cases continue to occur because the prohibition is not fully enforced and contains exceptions (eg, sales for bona fide scientific, educational, or exhibition purposes). Street vendors and flea markets are a common source of illegal sales.

This *S. typhimurium* outbreak is the third multistate, turtle-associated *Salmonella* outbreak in the United States since 2006. Before 2006, no large multistate turtle-associated *Salmonella* outbreaks were identified. One reason for this apparent increase might be PulseNet, which has improved the ability to detect multistate outbreaks. Increased pet turtle ownerships in the United States also might contribute to recent outbreaks.

Despite recommendations from Centers for Disease Control and Prevention to prevent turtle-associated salmonellosis in humans, recent outbreaks suggest that public education efforts have not been successful. Although many reptiles carry *Salmonella*, small turtles pose a greater risk to young children because they are perceived as safe pets, are small enough to be placed in the mouth, or otherwise can be handled inappropriately.

Direct or indirect reptile contact is associated with an estimated 6% of *Salmonella* infections in the United States and 11% of infections among persons younger than 21 years. Increasing enforcement of existing local, state, and federal regulations against the sale of small turtles, increasing penalties for illegal sales, and enacting more state and local laws regulating the sale of small turtles (eg, requiring *Salmonella* awareness education at the point of sale) could augment federal prevention efforts and facilitate a more rapid public health response.

Impact of HAART and CNS-penetrating antiretroviral regimens on HIV encephalopathy among perinatally infected children and adolescents

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Objectives: Prior to antiretroviral treatment, HIV infected children frequently devel oped encephalopathy, resulting in debilitating morbidity and mortality. This is the first large study to evaluate the impact of HAART and central nervous system (CNS) penetrating antiretroviral regimens on the incidence of HIV encephalopathy and survival after diagnosis of HIV encephalopathy among perinatally infected children.

Design: A total of 2398 perinatally HIV infected children with at least one neurological examination were followed in a US based prospective cohort study conducted from 1993 to 2007.

Methods: Trends in incidence rates over calendar time were described and Cox regression models were used to estimate the effects of time varying HAART and CNS penetrating antiretroviral regimens on HIV encephalopathy and on survival after diagnosis of HIV encephalopathy.

Results: During a median of 6.4 years of follow up, 77 incident cases of HIV ence phalopathy occurred [incidence rate 5.1 cases per 1000 person years, 95% confidence interval (CI) 4.0 6.3]. A 10 fold decline in incidence was observed beginning in 1996, followed by a stable incidence rate after 2002. HAART regimens were associated with a 50% decrease (95% CI 14 71%) in the incidence of HIV encephalopathy compared with non HAART regimens. High CNS penetrating regimens were associated with a substantial survival benefit (74% reduction in the risk of death, 95% CI 39 89%) after HIV encephalopathy diagnosis compared with low CNS penetrating regimens.

Conclusion: A dramatic decrease in the incidence of HIV encephalopathy occurred after the introduction of HAART. The use of HAART was highly effective in reducing the incidence of HIV encephalopathy among perinatally infected children and adolescents. Effective CNS penetrating antiretroviral regimens are important in affecting survival after diagnosis of HIV encephalopathy.

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Keywords: adolescent, antiretroviral therapy, children, HAART, HIV encephalopathy

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Introduction

The first pediatric cases of HIV encephalopathy were reported in 1985 among children with AIDS [1–3]. The prevalence of HIV encephalopathy among this population varied from 30 to 50% and latency to HIV encephalopathy ranged from 2 months to 5 years [3,4]. Clinical features of HIV encephalopathy include loss of or failure to attain developmental milestones, impaired brain growth, and motor deficits [1–4]. Some children present with rapidly progressive fatal disease, whereas others have stable phases interspersed with short periods of neurological deterioration [1–3]. Given the level of morbidity and mortality associated with this disease, HIV encephalopathy was added as an AIDS defining condition in 1987 [5].

Studies of cerebrospinal fluid (CSF) from children with HIV encephalopathy found active and persistent brain infection with HIV suggesting a need for antivirals that penetrate the blood–brain barrier [3,6]. Drug manufacturers and independent studies have evaluated the penetration of specific antiretroviral drugs into CSF [7]. To aid clinicians in antiretroviral therapy decision making for patients with neurological symptoms, this information has recently been used to develop a drug ranking system based on a drug's ability to penetrate the central nervous system (CNS) [7]. The clinical utility of these CNS penetration effectiveness ranks still needs to be validated in pediatric populations and clinical studies examining neurological outcomes such as HIV encephalopathy [7].

With yje advent of HAART, survival has increased [8,9]. The effect of such therapy on neurological disease such as HIV encephalopathy, however, is less clear. Several studies [10–13] have suggested a decreasing incidence of HIV encephalopathy. However, heterogeneity in the time scales and study populations used make it difficult to assess trends and attribute them to antiretroviral use.

The majority of studies examining the association between antiretroviral use and HIV encephalopathy [14–17] have focused on the effect of antiretroviral therapy on improving neuropsychological functioning after diagnosis of encephalopathy. One study [18] examined the effect of antiretroviral therapy before diagnosis of encephalopathy but restricted their study population to children with an eventual diagnosis of HIV encephalopathy. It found antiretroviral therapy to be associated with a later age at diagnosis. Another study compared 13 children ever diagnosed with HIV encephalopathy with 113 children never diagnosed with HIV encephalopathy and found a significantly greater proportion of HAART use among the children with a diagnosis of HIV encephalopathy [13]. The temporal relationship between HAART use and diagnosis is unclear in this study and no multivariate analyses were conducted to adjust for confounding by indication. Three recent studies [19–21] compared effect of early versus deferred antiretroviral therapy on AIDS progression and/or mortality and found a higher number of HIV encephalopathy cases among the groups that deferred therapy. Each of these studies, however, only had a few HIV encephalopathy cases (range 3–9 cases) to compare between their early and deferred treatment groups. No studies to date have quantified the effect of antiretroviral use on the risk of HIV encephalopathy. Our study describes the incidence of HIV encephalopathy between 1994 and 2006 among a cohort of perinatally HIV infected children enrolled in a large multicenter cohort study in the United States and evaluates the effects of HAART and CNS penetrating antiretroviral regimens on the incidence of encephalopathy. It further assesses the effects of HAART and CNS penetrating antiretroviral therapy on overall survival and survival after diagnosis of HIV encephalopathy.

Methods

The study population included participants from Pediatric AIDS Clinical Trials Group (PACTG) Protocols 219 and 219C, which were prospective studies designed to evaluate the long term effects of HIV infection and in utero and postnatal exposure to antiretroviral therapy. Between April 1993 and September 2000, infected and uninfected children from more than 80 study sites in the United States were eligible for enrollment in PACTG 219 if they were born to HIV infected mothers enrolled in PACTG perinatal trials or were themselves enrolled in PACTG perinatal or clinical trials and were younger than 21 years of age at entry. In September 2000, all children in PACTG 219 were encouraged to enroll into PACTG 219C, which expanded entry criteria to allow all HIV infected children at the study sites to enroll into the cohort. These studies were approved by the human subjects review boards at each participating institution and written informed consent was obtained from each child's parent or legal guardian. The population eligible for this study included 2398 perinatally HIV infected children enrolled in PACTG 219 and 219C between 1993 and 2006 who had at least one neurological examination.

At each study visit, data on sociodemographic characteristics, clinical diagnoses, antiretroviral therapies, and CD4 cell measurements were collected. HIV RNA measurements, however, were not routinely collected or available before 2000 and were available for only 46% of the population. We, therefore, could only adjust for HIV RNA viral load as a potential confounder in secondary analyses restricted to that subset. Baseline CD4 cell percentage and viral load were defined as the closest CD4 cell and HIV RNA measurement recorded either prior to or a week after the first neurological examination.

Neurological diagnoses reported on neurological exam ination and diagnoses forms were reviewed by the study pediatric neurologist, and dates of HIV encephalopathy diagnoses were confirmed. Only HIV encephalopathy diagnoses assumed to be progressive based on the best available information for review were included as cases.

HAART exposure was defined as the concomitant use of at least three drugs from at least two classes of HIV drugs. HIV drugs were classified into three main categories: nucleoside/nucleotide reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors. In analyses, children could switch from no HAART to HAART when they initiated a HAART regimen. However, for statistical simplicity, children were considered to remain on HAART for the length of follow up after HAART initiation. In the earlier PACTG 219 cohort, actual dates of initiation of medication or dates of changes in the use of medications were not available. Following previous evaluations of HAART using data from PACTG 219 [7–8], we assumed the midpoint between the visit date at which use of treatment was recorded and the date of the prior visit to be the date of treatment initiation.

To rank antiretroviral regimens according to their ability to penetrate the CNS, a modified version of the CNS penetration effectiveness rank developed by Letendre *et al.* [7] was used. The modification accounts for the total number of antiretroviral drugs in a regimen and has been used to predict change in CSF HIV RNA levels [22]. A scale of 1 (lowest penetration) to 3 (highest penetration) was used to rank each antiretroviral drug (Table 1). A CNS penetration score was calculated for each antire troviral regimen by summing the individual ranks of each antiretroviral drug included in the regimen. Antiretroviral regimens with scores less than 4 were classified as low CNS penetrating regimens, scores of 4–5 were classified as medium CNS penetrating regimens, and scores greater than or equal to 6 were classified as high CNS penetrating regimens. In analyses, children could switch from low CNS penetrating regimens to higher CNS penetrating regimens. However, for statistical simplicity, once a child initiated his/her highest CNS penetrating regimen, he/she was considered to remain on that regimen for the length of follow up.

The baseline date for all children was defined as the date of their first neurological examination. Prevalent cases of HIV encephalopathy were identified as of this baseline date and removed from subsequent analyses. However, age at diagnosis and subsequent mortality are presented descriptively for prevalent diagnoses. For the analyses of incident HIV encephalopathy, each child was followed from his/her baseline date to the date of HIV encephalopathy diagnosis, death, or his/her last visit before 31 May 2007 (date of closure of PACTG 219C), whichever came first. For overall survival analyses, each child was followed from his/her baseline date to date of death, or censored as of his/her last visit before study closure. For analyses of survival after HIV encephalopathy diagnosis, all children with an incident diagnosis of HIV encephalopathy were followed from their date of HIV encephalopathy diagnosis to their date of death or censored as of their last study visit. The rate of loss to follow up in the HIV infected children enrolled in the PACTG 219C study was 3–4% per year [23].

Extended Cox regression models were used to estimate the effects of time varying HAART versus no HAART on the incidence of HIV encephalopathy, overall survival, and survival after HIV encephalopathy diagnosis. Extended Cox regression models were also used to estimate the effects of time varying medium and high CNS penetrating regimens versus low CNS penetrating regimens on the incidence of HIV encephalopathy, overall survival, and survival after HIV encephalopathy diagnosis. Age at baseline, sex, race/ethnicity, birth weight, and baseline CD4 cell percentage were included as covariates in all models. In secondary analyses, extended Cox models were used to evaluate the effect of HAART and CNS penetrating regimens on HIV encephalopathy adjusting also for HIV RNA and to investigate the effect of HIV encephalopathy diagnosis on mortality. Analyses were conducted using SAS version 9 (SAS Institute, Cary, North Carolina, USA).

Results

Of the 3553 HIV infected children enrolled in PACTG 219 and 219C, 3193 were perinatally infected and 2398 of these (75%) had at least one neurological examination.

Table 1. Central nervous system penetration scale for antiretroviral drugs.

1 (lowest penetration)	2 (medium penetration)	3 (highest penetration)
Didanosine (ddI)	Emtricitabine (FTC)	Abacavir (ABC)
Tenofovir (TFV)	Lamivudine (3TC)	Zidovudine (ZDV)
Zalcitabine (ddC)	Stavudine (d4T)	Delavirdine (DLV)
Nelfinavir (NFV)	Efavirenz (EFV)	Nevirapine (NVP)
Ritonavir (RTV)	Amprenavir (APV)	Amprenavir/ritonavir (APV r)
Saquinavir (SQV)	Atazanavir (ATV)	Atazanavir/ritonavir (ATV r)
Saquinavir/ritonavir (SQV r)	Fosamprenavir (f APV)	Fosamprenavir/ritonavir (f APV r)
Tipranavir/ritonavir (TPV r)	Indinavir (IDV)	Indinavir/ritonavir (IDV r)
Enfuvirtide (T 20)		Lopinavir/ritonavir (LPV r)

The perinatally HIV infected children with a neurological examination were generally similar to the 795 without, although those excluded were more likely to be female children, of older age, and to have higher CD4 cell percentage levels. At baseline, 126 cases of HIV encephalopathy were identified among 2398 children with a neurological examination, resulting in a prevalence of 5.3% [95% confidence interval (CI) 4.4–6.2%]. The median age at diagnosis of these prevalent cases was 1.7 years (Q1, Q3: 0.9, 3.9). Twenty of the 126 prevalent cases (16%) had initiated HAART before diagnosis, 60 (48%) had initiated non HAART regimens, and 46 (36%) had not initiated any antiretroviral therapy before diagnosis of HIV encephalopathy. There were 42 deaths among the 126 prevalent cases with a median survival after diagnosis of 1.4 years (Q1, Q3: 0.7, 2.9). The majority of the deaths (62%) occurred among children who never initiated HAART during their lifetime.

The baseline characteristics of the 2272 children followed for incident HIV encephalopathy and overall survival analyses are provided in Table 2. Half of the children were female, 48% were less than or equal to 5 years of age at the time of their first neurological examination, 82% were born prior to 1995, 55% were black, 24% were classified as having low birth weight (<2500 g), and 19% had severe immunosuppression (CD4 cell <15%). Of the 1044 children with HIV RNA information at baseline, 16% had viral load of at least 100 000 copies/ml. At the time of their first neurological examination, 35% of the children were on a HAART regimen and 27% were on a high CNS penetrating regimen.

Over a median duration of 6.4 years of follow up, (Q1, Q3: 3.6, 9.9), 77 incident cases of HIV encephalopathy occurred, yielding an incidence rate of 5.1 cases per 1000 person years (95% CI 4.0–6.3, person years: 15 178). The median age at diagnosis of the 77 incident cases was 6.3 years (Q1, Q3: 3.3, 11.4). In Fig. 1, the incidence rates and the percentage of children on HAART in the study population are summarized from 1994–2006. Beginning in 1996, there was a 10 fold decrease in the incidence of HIV encephalopathy followed by a relatively stable incidence rate after 2002. Conversely, there was a significant increase in the percentage of children on HAART regimens after 1996, suggesting an impact of HAART on the decreased incidence of HIV encephalopathy over time. By the end follow up, 1806 children (79%) had initiated HAART and 31 of the 77 HIV encephalopathy cases (40%) were observed among them. Four hundred and sixty six children never initiated HAART with 46 of the 77 HIV encephalopathy cases (60%) observed among them. Of the 1741 children who had initiated a high CNS penetrating antiretroviral regimen by end of follow up, 34 (2%) had a diagnosis of HIV encephalopathy. Twenty four (9%) of the 267 children who ended follow up on a medium CNS penetrating regimen had a diagnosis of HIV encephalopathy, and 19 (7%) of the 264 children on a low CNS penetrating regimen had a diagnosis of HIV encephalopathy by end of follow up.

Table 2. Characteristics of study population followed for incident HIV encephalopathy and overall survival at time of first neurologic examination ($N = 2272$).

Characteristic	*N* (%)
Sex	
Male	1136 (50)
Female	1136 (50)
Age	
≤1 year	268 (12)
2 5 years	828 (36)
6 10 years	833 (37)
>10 years	343 (15)
Birth year	
<1990	874 (38)
1990 1994	996 (44)
≥1995	402 (18)
Race/ethnicity	
White, Non Hispanic[a]	342 (15)
Black, Non Hispanic	1247 (55)
Hispanic	683 (30)
Birth weight	
<2500 g	551 (24)
≥2500 g	1640 (72)
Unknown	81 (4)
CD4 cell percentage	
<15	433 (19)
15 24	505 (22)
≥25	1271 (56)
Missing	63 (3)
HIV RNA load (copies/ml)	
≤400	327 (14)
401 99 999	550 (24)
≥100 000	167 (7)
Missing	1228 (54)
Antiretroviral therapy	
HAART	786 (35)
Non HAART	1486 (65)
CNS penetrating regimens	
Low	896 (39)
Medium	765 (34)
High	611 (27)

CNS, central nervous system.
[a]Other race/ethnicity (*N* 36) included in White, Non Hispanic category.

Over a median follow up of 6.5 years (Q1, Q3: 3.9, 10) from the first neurologic examination until death or censoring, 207 deaths were reported, resulting in a mortality rate of 13.5 per 1000 person years (95% CI 11.7–15.4, person years = 15 389). By the end of follow up, 1826 children had initiated HAART and 101 deaths (6%) were observed among them. The number initiating HAART is higher than that reported above for the HIV encephalopathy incidence analysis as some children initiated HAART after their diagnosis. Four hundred and forty six children never initiated HAART and 106 deaths (24%) were observed among them. Of the 1756 children who had initiated a high CNS penetrating regimen, 111 (6%) had died by the end of follow up. Twenty six deaths (10%) were observed among 255

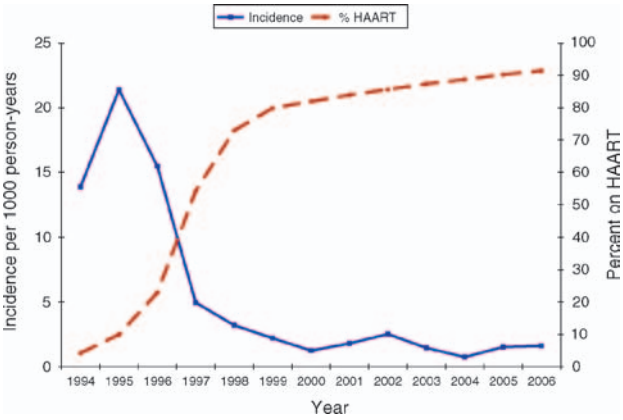

Fig. 1. Incidence of HIV encephalopathy and percentage of children on HAART from 1994 to 2006.

children on a medium CNS penetrating regimen and 70 deaths (27%) were observed among 261 children on a low CNS penetrating regimen.

The total person time accrued for the 77 incident cases followed for survival was 219 person years. Forty three deaths were observed during follow up resulting in a mortality rate of 196.3 per 1000 person years (95% CI 142.1–264.5). Median survival after diagnosis was 2.0 years (Q1, Q3: 0.1, –). By the end of follow up, 51 of the 77 children with a diagnosis of HIV encephalopathy had initiated HAART and 18 deaths were observed among them, as compared with 25 deaths among the 26 children who never initiated HAART. The numbers of deaths occurring by level of CNS penetrating regimen were 19, 11, and 13 among 50, 12, and 15 children ending follow up on a high, medium, or low penetrating regimen.

Children who initiated HAART had a 50% lower risk of developing HIV encephalopathy compared with those who were not on HAART (hazard ratio 0.50, 95% CI 0.29–0.86) (Table 3). Baseline CD4 cell values less than 15% were associated with a greater than eight fold increased risk of HIV encephalopathy (hazard ratio 8.41, 95% CI 4.79–14.76). Age less than or equal to 1 year at first neurologic examination was also independently associated with a greater than three fold increase in HIV encephalopathy with a hazard ratio of 3.38 (95% CI 1.36–8.44). In the secondary analysis restricted to the subset of the population with HIV RNA measurements, the HIV encephalopathy hazard ratio comparing HAART to no HAART was even stronger, 0.32 (95% CI 0.12–0.85) after adjustment for viral load. We also considered adjustment for calendar year, but treatment effects were attenuated and became nonsignificant due to the high correlation between calendar year and introduc tion of HAART (analyses not presented).

High CNS penetrating regimens, as defined by CNS penetrating scores, were associated with a 41% reduced incidence of HIV encephalopathy compared with low penetrating regimens, although this association was not statistically significant (hazard ratio 0.59, 95% CI 0.31–1.10; Table 3). After adjustment for viral load, the effect of high CNS penetrating regimens on incidence of HIV encephalopathy was even stronger compared with low CNS penetrating regimens (hazard ratio 0.41, 95% CI 0.13–1.29). Given the restricted sample size in this subanalysis, this large protective association was not statistically significant. Similar to the HAART analyses, these treatment effects were attenuated with adjustment for calendar year due to the correlation between calendar year and introduction of effective CNS penetrating regimens (analyses not presented).

In the overall population, HAART and effective CNS penetrating regimens were associated with increased survival compared with no HAART and low CNS penetrating regimens, respectively (Table 4). Children with a diagnosis of HIV encephalopathy, however, had 12 times the risk of death compared with children without a

Table 3. Estimated effects of HAART and central nervous system-penetrating antiretroviral regimens on incident HIV encephalopathy.

Characteristic	Hazard ratio[a] (95% CI)	*P*
Antiretroviral therapy		
HAART	0.50 (0.29, 0.86)	0.01
Non HAART	Referent	
CNS penetrating regimens		
Low	Referent	
Medium	0.86 (0.46, 1.62)	0.64
High	0.59 (0.31, 1.10)	0.10

CI, confidence interval; CNS, central nervous system.
[a]Multivariate hazard ratios adjusted for age, sex, race, birth weight, and CD4 cell percentage at baseline.

Table 4. Estimated effects of HAART and central nervous system-penetrating antiretroviral regimens on overall survival and survival after diagnosis of incident HIV encephalopathy.

Characteristic	Hazard ratio[a] (95% CI)	*P*
Effect on overall survival in the entire cohort (*N* 2272)		
Antiretroviral therapy		
HAART	0.41 (0.29, 0.58)	<0.0001
Non HAART	Referent	
CNS penetrating regimens		
Low	Referent	
Medium	0.25 (0.16, 0.40)	<0.0001
High	0.31 (0.22, 0.45)	<0.0001
Effect on survival after diagnosis of HIV encephalopathy (*N* 77)		
Antiretroviral therapy		
HAART	0.51 (0.25, 1.05)	0.07
Non HAART	Referent	
CNS penetrating regimens		
Low	Referent	
Medium	0.51 (0.20, 1.33)	0.17
High	0.26 (0.11, 0.61)	0.002

CI, confidence interval; CNS, central nervous system.
[a]Each adjusted for age, sex, race, birth weight, and CD4 cell percen tage.

diagnosis of encephalopathy (hazard ratio 12.42, 95% CI 8.46–18.24). Among the 77 children with an incident diagnosis of HIV encephalopathy, HAART use halved the risk of death after diagnosis compared with non HAART regimens (hazard ratio 0.51, 95% CI 0.25–1.05), but use of high CNS penetrating regimens conferred a larger survival benefit (74% reduction in risk of death) after HIV encephalopathy diagnosis compared with low CNS penetrating regimens (hazard ratio 0.26, 95% CI 0.11–0.61; Table 4).

Discussion

The present study describes a 10 fold decrease in the incidence of HIV encephalopathy among a large prospective cohort of perinatally infected children enrolled over a 14 year period, 1993–2006. This dramatic decline in incidence occurred with a concurrent increase in use of HAART, proposing an association between HAART use and risk of HIV encephalopathy. Although this hypothesis has been suggested by previous studies [13,24], this is the first study to quantify the impact of HAART on the incidence of HIV encephalopathy.

We found HAART use to decrease the risk of HIV encephalopathy by 50% compared with no HAART use. Similar to previous studies [8,9], we also found HAART use to be associated with improved overall survival compared with no HAART use. HAART also resulted in an improvement in survival after diagnosis of HIV encephalopathy, although this association was not statistically stable due to the small sample size of incident cases. These results suggest that HAART inhibits or delays HIV dissemination in the CNS and may also decrease viral replication if an active and persistent infection is already established in the brain.

One study found a significant decrease in CNS viral load with increasing numbers of CNS penetrating antiretroviral drugs independent of HAART alone [25]. We evaluated the effect of CNS penetrating regimens on the risk of HIV encephalopathy and found effective CNS penetrating regimens to be associated with a lower incidence of HIV encephalopathy, although not statistically significant. However, we did observe a significant survival benefit of using high CNS penetrating regimens after diagnosis of HIV encephalopathy. Optimal levels of antiretroviral drugs within the CNS, over what some HAART regimens provide, are likely needed to stop the active replication of HIV within the brain that can lead to further neurological decline and eventually death. Although the utility of effective CNS penetrating antiretroviral drugs is clear in improving survival after diagnosis of HIV encephalopathy, a study assessing the impact of such drugs on cognitive impairment was equivocal [26]. Further research is required to assess the impact of antiretroviral CNS penetration effectiveness on the various pathogenic mechanisms leading to neurological deterioration and disease.

A recent study evaluating the ability of the protease inhibitor atazanavir to penetrate the CNS [27] found cerebrospinal concentrations of atazanavir to be highly variable even when boosted with ritonavir. This result suggests a modification of the CNS penetration effectiveness ranks proposed by Letendre *et al.* [7]. Given that only 1% of the children classified as initiating a high CNS penetrating regimen in our study used atazanavir boosted with ritonavir, any future modification of the ranking system is unlikely to change our results.

Our study population only included children with at least one neurological examination in PACTG 219 or 219C. This inclusion criterion allowed identification of pre existing diagnoses of HIV encephalopathy by a pediatric neurologist and subsequent follow up after the first examination of confirmed 'disease free' children at risk for incident HIV encephalopathy. However, this inclusion criterion did exclude 795 perinatally infected children who did not have a neurological examination in PACTG 219 or 219C. This excluded population had significantly more female children and higher CD4 cell percentage compared with the study population. They also were significantly older than the study population. Although sex was not associated with risk of HIV encephalopathy in our analyses, the associations of age and CD4 cell percentage with risk of HIV encephalopathy suggest that the excluded population would have a lower risk of HIV encephalopathy compared with the study population.

The children in our study population were not followed from birth. Therefore, early fatal cases of HIV encephalopathy may have been missed leading to an underestimation of the incidence of HIV encephalopathy. The median age of the incident cases of HIV encephalopathy identified during follow up was also greater than the median age of the prevalent cases of HIV encephalopathy identified at baseline, suggesting that a 'survivor' cohort of children was followed for incident analyses. If these 'survivors' were at lower risk of HIV encephalopathy compared with the general pediatric HIV infected population, then the estimated incidence rates of HIV encephalopathy may also be underestimated. Our interest in the trend in incidence of HIV encephalopathy over time, however, would not be affected by this potential survivor effect.

As viral load information was missing for 46% of the study population, we were not able to adjust for this important confounder in our primary analyses. Sensitivity analyses conducted among the subset of children with viral load information suggest that stronger protective effects of HAART and high CNS penetrating regimens on risk of HIV encephalopathy and survival after diagnosis of HIV encephalopathy would have been estimated with

adjustment of viral load. However, as clinicians did not have viral load information to make treatment decisions in the early years of follow up, viral load could not be a confounder in these time periods.

Diagnoses of HIV encephalopathy were collected in the study population either from chart abstraction or from neurological examination forms. It is unclear whether diagnoses collected from chart abstraction were made by treating clinicians or pediatric neurologists. Given the wide spectrum of neurological disease experienced by perinatally infected children, there may be some outcome misclassification of HIV encephalopathy. Assuming that this misclassification is nondifferential with respect to treatment, our observed protective effects of HAART and CNS penetrating antiretroviral regimens on risk of HIV encephalopathy and survival after diagnosis of HIV encephalopathy, respectively, are conservative estimates.

In conclusion, HAART use was highly effective in reducing the risk of HIV encephalopathy among perinatally infected children and adolescents. Among children with a diagnosis of HIV encephalopathy, treatment decisions should consider the effectiveness of antiretroviral drugs in penetrating the CNS. Highly CNS penetrating regimens conferred a substantial survival benefit to children with HIV encephalopathy compared with low CNS penetrating regimens. Of course, any potential toxicity of individual or combination antiretroviral drugs [28] must be weighed with their ability to penetrate the CNS when making treatment decisions in pediatric HIV infected populations.

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Early Antiretroviral Therapy and Mortality among HIV-Infected Infants

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Abstract

Background—In countries with a high seroprevalence of human immunodeficiency virus type 1 (HIV-1), HIV infection contributes significantly to infant mortality. We investigated antiretroviral-treatment strategies in the Children with HIV Early Antiretroviral Therapy (CHER) trial.

Methods—HIV-infected infants 6 to 12 weeks of age with a CD4 lymphocyte percentage (the CD4 percentage) of 25% or more were randomly assigned to receive antiretroviral therapy (lopinavir–ritonavir, zidovudine, and lamivudine) when the CD4 percentage decreased to less than 20% (or 25% if the child was younger than 1 year) or clinical criteria were met (the deferred antiretroviral-therapy group) or to immediate initiation of limited antiretroviral therapy until 1 year of age or 2 years of age (the early antiretroviral-therapy groups). We report the early outcomes for infants who received deferred antiretroviral therapy as compared with early antiretroviral therapy.

Results—At a median age of 7.4 weeks (interquartile range, 6.6 to 8.9) and a CD4 percentage of 35.2% (interquartile range, 29.1 to 41.2), 125 infants were randomly assigned to receive deferred therapy, and 252 infants were randomly assigned to receive early therapy. After a median follow-up of 40 weeks (interquartile range, 24 to 58), antiretroviral therapy was initiated in 66% of infants in the deferred-therapy group. Twenty infants in the deferred-therapy group (16%) died versus 10 infants in the early-therapy groups (4%) (hazard ratio for death, 0.24; 95% confidence interval [CI], 0.11 to 0.51; P<0.001). In 32 infants in the deferred-therapy group (26%) versus 16 infants in the early-therapy groups (6%), disease progressed to Centers for Disease Control and Prevention stage C or severe stage B (hazard ratio for disease progression, 0.25; 95% CI, 0.15 to 0.41; P<0.001). Stavudine was substituted for zidovudine in four infants in the early-therapy groups because of neutropenia in three infants and anemia in one infant; no drugs were permanently discontinued. After a review by the data and safety monitoring board, the deferred-therapy group was modified, and infants in this group were all reassessed for initiation of antiretroviral therapy.

Conclusions—Early HIV diagnosis and early antiretroviral therapy reduced early infant mortality by 76% and HIV progression by 75%. (ClinicalTrials.gov number, NCT00102960.)

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Infants with human immunodeficiency virus type 1 (HIV-1) infection have higher rates of disease progression and mortality than older children,[1-3] even with a high percentage of CD4 lymphocytes (CD4 percentage).[4] Whereas early initiation of antiretroviral therapy may be appropriate for infants, continuing treatment for life is problematic, given the limitations of the available drugs, the long-term toxicity of antiretroviral therapy, adherence issues, the risk of resistance to antiretroviral therapy, and limited resources. This trial addresses the optimal time of initiation and duration of antiretroviral therapy in infants with in utero or intrapartum HIV-1 infection. We hypothesized that early initiation of limited antiretroviral therapy soon after primary infection, when the immune system is most immature, would be beneficial and would delay the time to initiation of continuous antiretroviral therapy. In accordance with the recommendation, in June 2007, of the the data and safety monitoring board, we report the early outcomes for infants who were randomly assigned to receive deferred therapy as compared with those assigned to receive early antiretroviral therapy.

Methods

Study Design

The Children with HIV Early Antiretroviral Therapy (CHER) trial is a phase 3, randomized, open-label trial conducted by the Comprehensive International Program for Research in AIDS — South Africa in collaboration with the Medical Research Council Clinical Trials Unit, United Kingdom, and the Division of AIDS (DAIDS) of the National Institutes of Health (NIH). The study is being conducted in two centers in South Africa: the Perinatal HIV Research Unit, Chris Hani Baragwanath Hospital, in Soweto, and the Children's Infectious Diseases Clinical Research Unit, Tygerberg Children's Hospital, in Cape Town.

We enrolled infants 6 to 12 weeks of age who had HIV infection (defined by a positive polymerase-chain-reaction [PCR] test for HIV-1 DNA and a plasma HIV-1 RNA level on PCR of >1000 copies per milliliter) and a CD4 percentage of 25% or more. Exclusion criteria are listed in the Supplementary Appendix, available with the full text of this article at www nejm.org. Infants were randomly assigned to receive one of three treatments: early limited antiretroviral therapy for 96 weeks, early limited antiretroviral therapy for 40 weeks, or deferred therapy. Immunologic criteria for initiating antiretroviral therapy in the deferred-therapy group or reinitiating antiretroviral therapy in the early-therapy groups were a CD4 percentage of less than 20%[5] or, in the case of children younger than 12 months, a CD4 percentage of less than 25% or a CD4 count of less than 1000 cells per cubic millimeter, according to World Health Organization (WHO) guidelines updated in 2006.[6] Clinical criteria for initiating or reinitiating antiretroviral therapy[7] were Centers for Disease Control and Prevention (CDC) stage C or investigator-selected (severe) stage B events (see the Supplementary Appendix), including symptomatic lymphoid interstitial pneumonitis, bronchiectasis, nephropathy, cardiomyopathy, and failure to thrive. GlaxoSmithKline provided lamivudine and zidovudine, and the South African Department of Health provided lopinavir–ritonavir. Written informed consent was obtained from the parents or legal guardians of all the infants. The authors vouch for the completeness and accuracy of the data.

Study Treatment

First-line antiretroviral therapy consisted of zidovudine at a dose of 240 mg per square meter of body-surface area twice daily and lamivudine at a dose of 4 mg per kilogram of body weight twice daily, with lopinavir–ritonavir given at a dose of 300 mg of lopinavir plus 75 mg of ritonavir per square meter twice daily until 6 months of age,[8] then at a dose of 230 mg of lopinavir plus 57.5 mg of ritonavir per square meter twice daily. The second-line regimen was predefined and consisted of didanosine, abacavir, and nevirapine (or efavirenz instead of

nevirapine if the child was older than 3 years of age). The criteria for switching to second-line therapy are listed in the Supplementary Appendix.

Screening, Randomization, and Follow-up

Infants who were exposed to HIV were identified from programs for the prevention of mother-to-child transmission of HIV-1 in the Western Cape and Gauteng provinces. PCR for HIV-1 DNA was performed from 4 weeks of age when cotrimoxazole prophylaxis commenced. In Gauteng, the regimen for the prevention of mother-to-child transmission of HIV-1 was single-dose nevirapine administered to both the mother and the neonate; in the Western Cape, the regimen was zidovudine administered to mothers from 34 weeks' gestation and to neonates for 7 days and single-dose nevirapine administered to both.

The randomization schedule was prepared centrally by the trial statistician and faxed to the study sites. Randomization was stratified according to clinical center, and blocks, varying randomly in size, were used to ensure balance in the number of infants assigned to each group according to center.

After randomization, the infants were seen every 4 weeks until week 24, then every 8 weeks until week 48, and every 12 weeks thereafter. At each scheduled visit, the evaluation included documentation of any HIV-related clinical events, a complete blood count with a differential count, measurements of aminotransferase levels, and a CD4 cell count with calculation of the CD4 percentage. The pharmacist at each site measured adherence to the assigned medication by comparing the amount of drug dispensed with that returned at the subsequent visit. Toxicity was graded according to the criteria of the DAIDS[9] of the National Institute of Allergy and Infectious Diseases (NIAID), at the NIH. The study was also conducted under a Food and Drug Administration investigational-new-drug application (number 71,494).

Outcomes

The primary outcome was the time to death or failure of the first-line antiretroviral therapy. The latter was defined as any of the following: failure to reach a CD4 percentage of 20% or more by week 24 of therapy or a decrease in the CD4 percentage to less than 20% after the first 24 weeks of antiretroviral therapy (immunologic failure), severe CDC stage B or stage C events (clinical failure), or toxicity requiring more than one drug substitution within the same class or a switch to a new class or requiring permanent discontinuation of treatment (i.e., regimen-limiting toxicity failure). An independent end-point review committee reviewed all deaths and CDC stage C and severe stage B events without knowledge of CD4 values, status of antiretroviral therapy, or randomized treatment assignments. Investigators at each of the two centers remained unaware of the primary and secondary outcomes at the other center.

Review and Monitoring

The trial was approved by the ethics committees of both participating institutions. According to the protocol, the study is reviewed at least annually by the independent DAIDS international data and safety monitoring board of Africa; this board can recommend termination or modification of the study because of safety or efficacy concerns. The guiding statistical criterion for “proof beyond reasonable doubt” is based on a difference of at least 3 SD in the log relative hazard (or nominal $P<0.001$) in any interim analysis (according to the Haybittle-Peto rule). Two such annual reviews have been conducted. In addition, a subcommittee of the data and safety monitoring board reviews all deaths according to randomized treatment assignments and can call for an unscheduled review by the full data and safety monitoring board if there are any safety concerns; the subcommittee has performed three reviews (approximately every 3 months).

At the second annual review of the data and safety monitoring board, in June 2007, by which time accrual had been completed, strong evidence of a difference in mortality emerged between infants who had been randomly assigned to receive early antiretroviral therapy and those assigned to the deferred-therapy regimen. The data and safety monitoring board recommended dissemination of these early findings and urgent evaluation of children in the deferred-therapy group who were not receiving antiretroviral therapy for possible initiation of antiretroviral therapy. The board also recommended that all three groups be continued, with modification in the deferred-therapy group as mentioned above.

Statistical Analysis

The sample size was estimated with the use of the methods previously developed for sample size and estimation of statistical power in complex clinical trials.[10,11] We assumed that the cumulative probability of death or regimen failure in the deferred-therapy group would be 0.06, 0.17, 0.28, 0.39, and 0.49 by years 1, 2, 3, 4, and 5, respectively. We assumed that for the first 5 years of follow-up, the annual hazard ratio for disease progression or death would be 0.51, 0.57, 0.78, 0.85, and 0.88 in the group that received early limited antiretroviral therapy for 40 weeks and 0.51, 0.27, 0.43, 0.67, and 0.74 in the group that received early limited antiretroviral therapy for 96 weeks, as compared with the deferred-therapy group. These assumptions were based on estimates of death rates, time to initiation of antiretroviral therapy, and time to failure of antiretroviral therapy in other pediatric cohorts.[4,12] Under these assumptions, the planned sample size of 375 children (125 per group), enrolled over a period of 18 months and followed for a minimum of 3.5 years, would provide 80% power to reject the null hypothesis of no difference among the three groups in the time to the primary outcome, on the basis of a global log-rank test with a two-sided alpha level of 0.05.

The planned primary analysis was to first test the null hypothesis of no difference among the three groups in the time to death or regimen failure by means of a global log-rank test with two degrees of freedom (with stratification according to site). If the null hypothesis was rejected, then each of the early-therapy groups was to be compared with the deferred-therapy group in terms of the average hazard during the follow-up period with the use of a Cox proportional-hazards model, also stratified according to site. Although pairwise comparisons of all groups were performed, we report the comparison of the deferred-therapy group with the combined early-therapy groups, preserving blinding between the early-therapy groups, as recommended by the data and safety monitoring board. All analyses are based on complete data as of June 20, 2007.

We used the intention-to-treat approach to compare the early-therapy groups combined with the deferred-therapy group. Time-to-event methods (i.e., Kaplan-Meier plots and the log-rank test stratified according to site) were used to compare the two groups for the time to the primary end point and survival. Cox proportional-hazards modeling was used to estimate a summary hazard ratio for death or treatment failure for the early-therapy groups combined as compared with the deferred-therapy group. The frequency of grade 3 or 4 adverse events in the two groups was compared with the use of a chi-square test. The time to initiation of continuous antiretroviral therapy in the deferred-therapy group was estimated with the use of Kaplan-Meier methods. Changes in the CD4 percentage and the CD4 count over time were summarized with the use of point estimates of mean changes from baseline documented at each visit. All reported P values are two-sided and have not been adjusted for multiple testing.

Results

Of 5985 infants born to mothers in programs for the prevention of mother-to-child transmission of HIV-1, 405 were HIV-positive (6.8%). The transmission rate was higher in Soweto (8.4%) than in Cape Town (5.6%), probably because of different regimens for the prevention of

mother-to-child transmission of HIV-1. An additional 155 HIV-infected infants were referred from other infant diagnosis programs. Among 560 HIV-infected infants considered for the trial, 110 were ineligible because the CD4 percentage was less than 25%, and 45 were ineligible for other reasons (Fig. 1). Between August 2005 and February 2007, a total of 377 infants were enrolled; 125 infants were randomly assigned to the deferred-therapy group, and 252 infants were randomly assigned to the early-therapy groups. Two pairs of twins were enrolled: the first pair underwent randomization, and the second pair of twins were nonrandomly assigned to the same group.

The median follow-up as of June 20, 2007, was 40 weeks (interquartile range, 24 to 58). Baseline characteristics were well matched in the study groups (Table 1). A total of 14% of the infants were breast-fed. Most mothers (62%) and infants (51%) received single-dose nevirapine for the prevention of mother-to-child transmission of HIV-1; 20% of the mothers and 27% of the infants received zidovudine and nevirapine. A smaller proportion (11% of the mothers and 16% of the infants) received no prophylaxis for the prevention of mother-to-child transmission of HIV-1.

Follow-up

Treatment was not completed in 14 infants in the early-therapy groups (6%) and in 4 infants in the deferred-therapy group (3%). The reasons were withdrawal of consent, for three infants in the early-therapy groups (1.2%) and for one infant in the deferred-therapy group (0.8%), and loss to follow-up, for the other 14 infants.

In the early-therapy groups, infants received antiretroviral therapy for 99% of the follow-up period, and in the deferred-therapy group, infants received antiretroviral therapy for 35% of the follow-up period. Eighty-three children in the deferred-therapy group (66%) started antiretroviral therapy according to the protocol criteria (63% met immunologic criteria and 36% met clinical criteria); 51 of these children (41%) were younger than 26 weeks of age. The estimated median time to the initiation of antiretroviral therapy in the deferred-therapy group was 21.1 weeks (Fig. 2). The adherence rate, defined as the percentage of drug received, was 87.8% for zidovudine, 90.2% for lamivudine, and 92.1% for lopinavir–ritonavir.

Mortality and Failure of the First Regimen

Thirty-two infants reached the primary end point of death or failure of the first regimen: 11 of 252 infants in the early-therapy groups combined and 21 of 125 in the deferred-therapy group (hazard ratio for early therapy as compared with deferred therapy, 0.25; 95% confidence interval [CI], 0.12 to 0.51). A total of 30 of 32 primary end points were deaths: 10 of 252 infants in the early-therapy groups (4%) died and 20 of 125 infants in the deferred-therapy group (16%) died (hazard ratio, 0.24; 95% CI, 0.11 to 0.51; $P<0.001$) (Fig. 3A and Table 2). Average rates of death were 5 per 100 person-years in the early-therapy groups and 21 per 100 person-years in the deferred-therapy group (Table 2). The death rate was much higher in the first 26 weeks after randomization and declined thereafter in both groups (Table 2); 20 of 30 infants died before 26 weeks of age. Fifteen infants in the deferred-therapy group died before receiving antiretroviral therapy; the other 5 infants received antiretroviral therapy, but death occurred within 1 month after initiation of antiretroviral therapy in four of five infants.

A total of 12 children (40%) died at home: 8 in the deferred-therapy group (6.4%) versus 4 in the early-therapy groups (1.6%); most deaths were unexpected and rapid. The cause of death was determined in two patients: one had gastroenteritis and one had disseminated tuberculosis. The cause of death was not determined in the remaining 10 infants. Hospital deaths were due to gastroenteritis (in five infants in the deferred-therapy group [4%] vs. four infants in the early-therapy groups [1.6%]), pneumonia (in four infants in the deferred-therapy group),

Pneumocystis jiroveci pneumonia (in two infants in the deferred-therapy group), cytomegalovirus (CMV) (in one infant in the deferred-therapy group), liver failure (granulomatous steatohepatitis detected on postmortem examination in one infant in an early-therapy group), and the sudden infant death syndrome (in one infant in an early-therapy group).

HIV Disease Progression

Disease progression occurred in 16 infants in the early-therapy groups (6.3%), as compared with 32 infants in the deferred-therapy group (25.6%) (hazard ratio, 0.25; 95% CI, 0.15 to 0.41; P<0.001) (Table 3). *P. jiroveci* pneumonia, CMV disease, and esophageal candidiasis occurred only in the deferred-therapy group. Four children had *P. jiroveci* pneumonia (two cases were definitive and two were presumptive). Failure to thrive was the most common event in both the deferred-therapy group and the early-therapy groups. The time to death or of the first CDC stage C or severe stage B event is shown in Figure 3B. Forty-one infants in the early-therapy groups were hospitalized (16.3%), as compared with 46 infants in the deferred-therapy group (36.8%) (Table 3).

Change in the CD4 Percentage during Follow-up

The mean changes from baseline in the CD4 percentage were as follows: at 12 weeks, +4.8% in the early-therapy groups versus −7.5% in the deferred-therapy group (absolute difference between the early-therapy groups and the deferred-therapy group, 12.3%; P<0.001); at 24 weeks, +5.9% versus −5.6% (absolute difference, 11.5%, P<0.001); at 32 weeks, +4.5% versus −4.8% (absolute difference, 9.3%; P<0.001); by week 40, when antiretroviral therapy had been initiated in more infants in the deferred-therapy group, the between-group difference had decreased to 6.7%.

Grade 3 and 4 Drug-Related Events

Of 58 grade 3 or 4 laboratory abnormalities, 20 were related to antiretroviral therapy and occurred in 19 children (Table 3). Fifteen of these children were in the early-therapy groups, and four were in the deferred-therapy group. In the early-therapy groups, neutropenia occurred in 10 children, anemia in 3, and elevated aminotransferase levels in 2. In the deferred-therapy group, three infants had neutropenia and one had elevated aminotransferase levels. Four children, all in the early-therapy groups, switched from zidovudine to stavudine because of neutropenia (in three children) or anemia and neutropenia (in one). No other drugs were discontinued.

Discussion

These data show that antiretroviral therapy initiated at a median age of 7 weeks reduced early mortality from 16% to 4% as compared with antiretroviral therapy initiated according to a threshold CD4 percentage or clinical progression of HIV disease; this is a relative reduction of 76%. A rapid decrease in CD4 values, rapid disease progression, and sudden death were all evident among infants in the deferred-therapy group, despite very close follow-up and regular CD4 monitoring. Although 66% of infants in this group received antiretroviral therapy, mainly because of a decreasing CD4 percentage, excess deaths could not be prevented. Another, smaller South African trial showed similarly rapid decreases in the CD4 percentage, with 85% of infants meeting the criteria for initiation of antiretroviral therapy (i.e., CD4 percentage <20%) by 6 months of age.[14]

More than one third of deaths in our study occurred at home, before caregivers recognized the need for medical attention. Mortality was consistently higher among infants in the deferred-therapy group than among those in the early-therapy groups throughout the first year of life, although differences were greatest among the youngest infants. Despite the randomization of

only asymptomatic infants with high CD4 percentages, the majority of deaths occurred within 6 months after randomization. In 27 of the 30 deaths, there were no antecedent CDC stage C or severe stage B events, and the infants died rapidly from the first significant clinical event, most commonly gastroenteritis or pneumonia. Deaths due to gastroenteritis occurred twice as frequently in the deferred-therapy group as in the early-therapy groups, suggesting that early antiretroviral therapy has a protective effect in infants in whom replacement feeding is common. Whether breast-feeding would have an additional beneficial effect remains to be investigated. Safe infant-feeding practices were promoted, irrespective of the feeding choice. Failure to thrive was twice as frequent in the deferred-therapy group as in the early-therapy groups; however, the occurrence of failure to thrive in infants receiving antiretroviral therapy underscores the importance of nutritional, caregiving, and social factors in these patients.

Although the logistics of HIV testing in early infancy were considerable, the low mother-to-child transmission rate (6.8%) in field conditions highlights the success of programs for the prevention of this type of transmission in the areas where the study was conducted. The timing of HIV infection — in utero or during the perinatal period — is unknown, since the first PCR test was performed at 4 weeks of age. The fact that the majority of the children became infected despite the use of regimens for the prevention of mother-to-child transmission suggests that many of the infections were acquired in utero and that such infections may have contributed to the high rates of rapid progression.[15] More rapid disease progression has also been reported among infants infected with HIV despite the use of regimens to prevent mother-to-child transmission of HIV-1 in countries with sufficient resources.[16] Our sample size limits comparisons of HIV disease progression among infants in the deferred-therapy group according to whether or not they had received a regimen for the prevention of mother-to-child transmission of HIV-1.

Luzuriaga et al. found better HIV suppression in infants who began to receive antiretroviral therapy before 3 months of age than in infants who began to receive this therapy later.[17] A number of U.S. and European cohort studies have reported better outcomes in infants receiving early antiretroviral therapy. However, interpretation of the data may be biased by the lack of randomization.[18-21] The HIV Paediatric Prognostic Markers Collaborative Study (HPPMCS),[4] a meta-analysis of data from untreated HIV-infected children in the United States and Europe, showed a risk of death that was increased by a factor of six for a 1-year-old child with high CD4 values as compared with a child 5 years of age with high CD4 values. The Cross Continents Collaboration for Kids study of untreated HIV-infected African children showed a poorer predictive value of CD4 values in young children after infancy and much higher mortality rates than those reported in the HPPMCS.[22] Current guidelines recommend the initiation of antiretroviral therapy on the basis of a low CD4 percentage or count, a high viral load, or the presence of clinical symptoms, whereas the treatment of asymptomatic infants with high CD4 values is not mandated. Results from the CHER trial provide support for early antiretroviral therapy to reduce mortality among infants who acquire HIV infection despite regimens for the prevention of mother-to-child transmission of HIV-1.[6,23-25]

In the CHER trial, infants received a protease inhibitor-based regimen as first-line antiretroviral therapy, according to South-African guidelines. A nevirapine-based regimen may not be advisable for early treatment in infants exposed to single-dose nevirapine.[26] Since guidelines for most resource-limited countries recommend starting antiretroviral therapy with nevirapine, the implementation of early antiretroviral therapy may pose considerable challenges. The estimated probability of death in the deferred-therapy group (17%) is lower than the 35% probability reported in African birth cohorts before the introduction of antiretroviral therapy or widespread cotrimoxazole prophylaxis.[3] By comparison, early natural-history studies in the United States and Europe showed mortality that was lower but rates of disease progression that were similar to the rates among infants in the deferred-therapy group in our study.[2,27]

These data provide strong support for the initiation of antiretroviral therapy from an early age, regardless of the CD4 percentage or count. A good program for the prevention of mother-to-child transmission of HIV-1, encompassing early diagnosis in infants, is fundamental to the success of any early antiretroviral-therapy strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

The following investigators, clinical centers, and committees participated in the Children with HIV Early Antiretroviral Therapy (CHER) trial: *South Africa* — A. Violari, J. McIntyre, W. Pelser, J. Steyn, S. Madhi, A. Naeem-Sheik, M. Budge, M. Saleh, S. Cassimjee, E. Lazarus, S. Mashinini, S. Dlamini, V. Kemese, J. Bolton (Perinatal HIV Research Unit, University of the Witwatersrand, Johannesburg); M.F. Cotton, H. Rabie, A. Janse van Rensburg, E. Dobbels, G. Fourie, M. Bester, W. Orange, R. Arendze, C. Andrea, M. Smuts, K. Smith, T. Louw, A. Abrahams, K. Kelly, A. Bohle, I. Mong, J. Howard, T. Cyster, G. Solomon, G. Benjamin, J. Mkalipi, E. Barnes (Children's Infectious Diseases Clinical Research Unit, Stellenbosch University, Tygerberg); I. Sanne, G. Gray, R. Panchia, C. Davies, M. Cornell (Comprehensive International Program for Research in AIDS — South Africa [CIPRA-SA]); W. Stevens, D. Glencross (CIPRA-SA Laboratory Core); S. Spector, C. van der Horst (CIPRA-SA Scientific Advisory Committee); *United Kingdom* — A.G. Babiker, D.M. Gibb, (Medical Research Council, Clinical Trials Unit [MRC CTU], London) J.-M. Steens, W.X. Snowden, N. Thoofer, E. Loeliger (Glaxo-SmithKline); *United States* — E. Handelsman, K. Reese, P. Jean-Phillipe, J. Nadler (Division of Acquired Immunodeficiency Syndrome, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]), J. McNamara (Division of Allergy, Immunology, and Transplantation, NIAID, NIH), R. Hoff (Regional Emerging Diseases Intervention Center), S. Lehrman (Merck), C. Oster (Walter Reed Army Institute of Research). **End Point and Clinical Events Review Committee:** T. Peto (chair) (John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom); L. Levin (Right to Care, Johannesburg); D. Gibb (MRC CTU, London). **Data Safety and Monitoring Board:** S. Ellenberg (chair) (University of Pennsylvania, Philadelphia); R. DerSimonian (executive secretary); A. Dodoo (University of Ghana, Accra); D. Harrington (Harvard School of Public Health, Boston); A. Kamali (MRC Uganda, Entebbe); E. Katabira (Makerere University Uganda, Kampala); C. Lombard (MRC South Africa, Cape Town); C. Luo (UNICEF, New York); M.F. Marshall (University of Minnesota, Minneapolis); L. Mokgatlhe (University of Botswana, Gaborone); A. Mwinga (Centers for Disease Control Zambia, Lusaka); A. Nunn (MRC CTU, London); H. Saloojee (University of the Witwatersrand, Johannesburg); M. Sande

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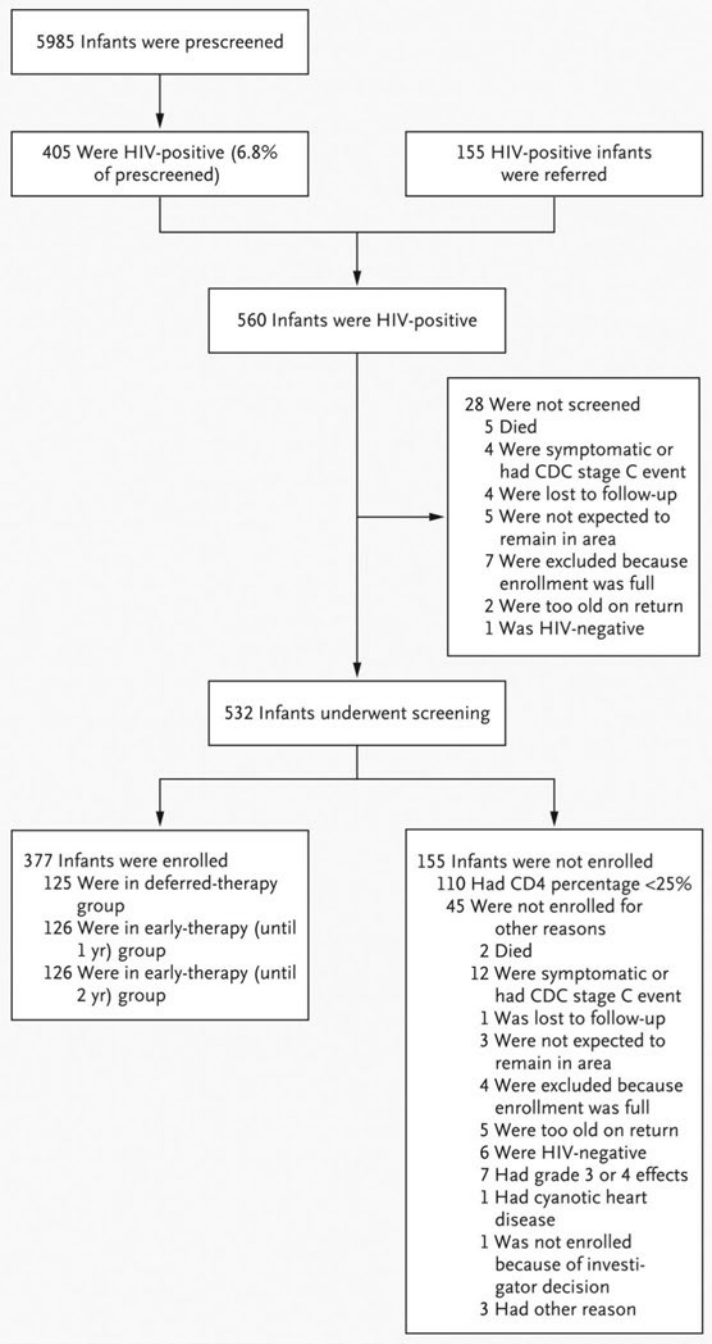

Figure 1.
Screening and Enrollment.

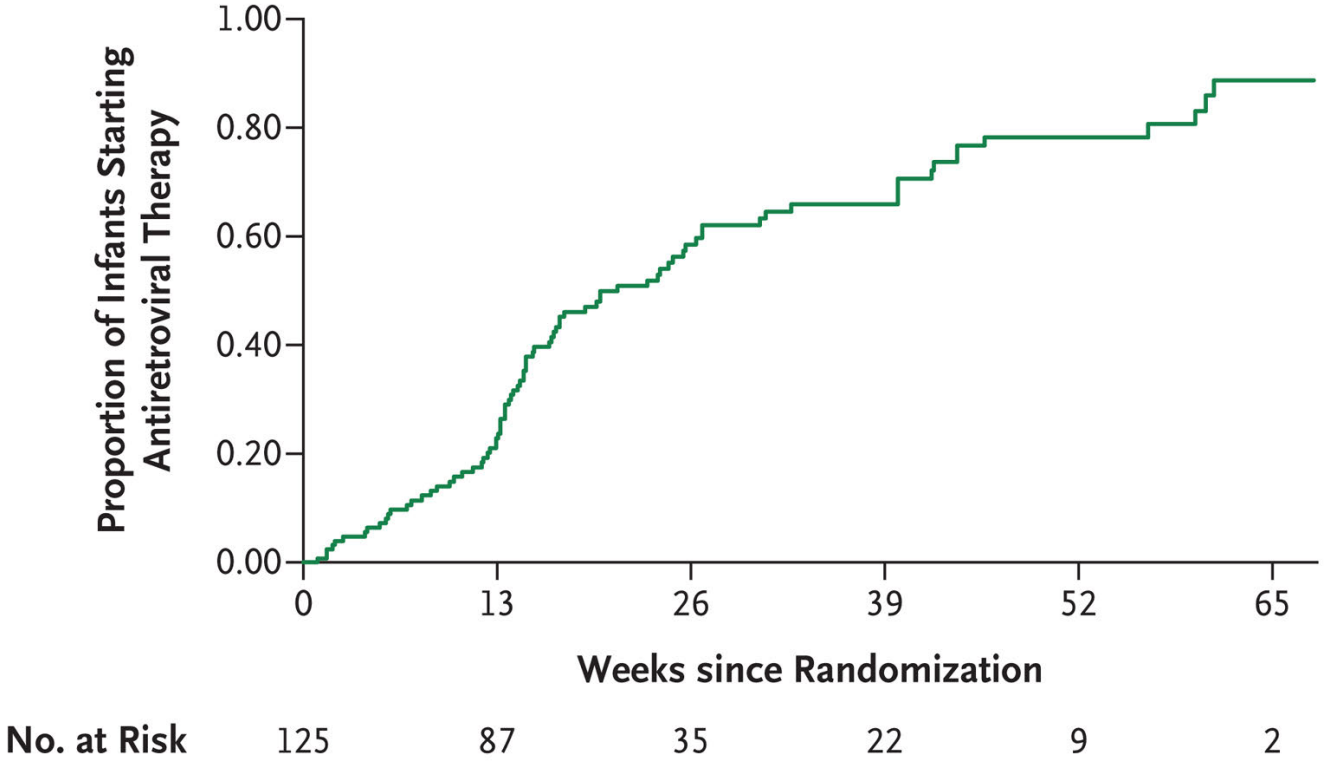

Figure 2.
Time to Initiation of Antiretroviral Therapy in the Deferred-Therapy Group.

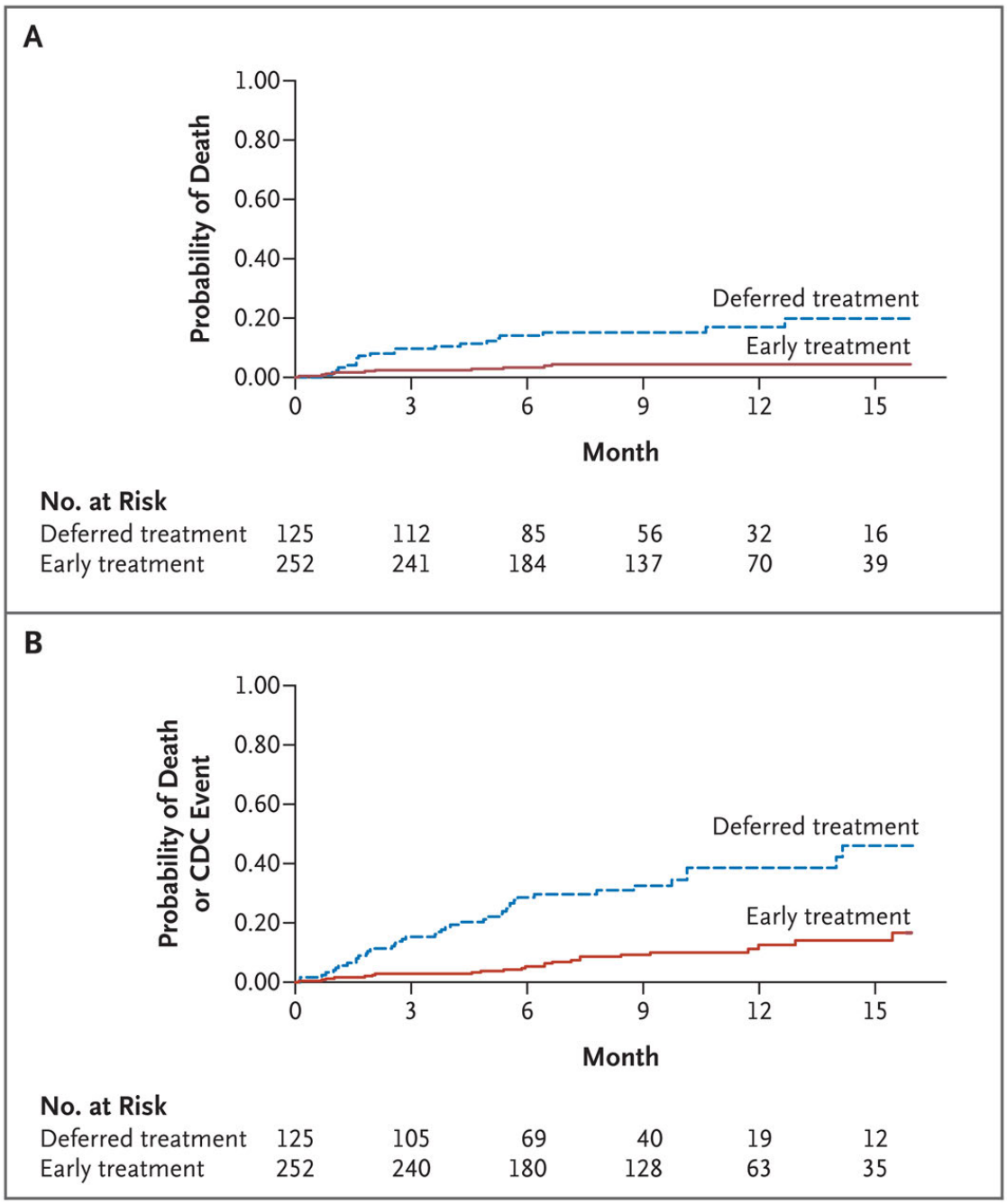

Figure 3. Probability of Death or a First Event, According to Treatment Group

Panel A shows the probability of death. Panel B shows the probability of death or the onset of a CDC stage C or severe stage B event.

Table 1

Baseline Characteristics of Infants Enrolled in the Trial.*

Variable	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)
Female sex — no. (%)	147 (58.3)	74 (59 2)	221 (58.6)
Age — wk			
Median	7.4	7.1	7.4
Interquartile range	6.6 to 8.9	6.4 to 8.9	6.6 to 8.9
Maternal antiretroviral therapy for prevention of mother-to-child transmission of HIV-1 — no. (%)			
No therapy	26 (10.3)	15 (12.0)	41 (10.9)
Nevirapine	162 (64.3)	72 (57.6)	234 (62.1)
Zidovudine	8 (3.2)	5 (4.0)	13 (3.4)
Zidovudine plus nevirapine	51 (20.2)	26 (20.8)	77 (20.4)
Maternal therapy with highly active antiretroviral therapy — no. (%)	2 (0.8)	5 (4.0)	7 (1 9)
Infant antiretroviral therapy for prevention of mother-to-child transmission of HIV-1 — no. (%)			
No therapy	40 (15.9)	20 (16.0)	60 (15.9)
Nevirapine	124 (49.2)	68 (54.4)	192 (50.9)
Zidovudine	5 (2.0)	1 (0.8)	6 (1.6)
Zidovudine plus nevirapine	69 (27.4)	34 (27 2)	103 (27.3)
Breast-fed infants — no. (%)	35 (13.9)	19 (15 2)	54 (14.3)
Weight — kg			
Median	4.4	4.5	4.4
Interquartile range	4.0 to 4.9	4.0 to 5.0	4.0 to 4.9
Weight for age — z score			
Median	−0.8	−0.5	−0.7
Interquartile range	−1.5 to 0.0	−1.4 to 0 2	−1.5 to 0.0
Weight for length — z score			
Median	0.3	0.3	0.3
Interquartile range	0.2 to 0.3	0.2 to 0.3	0.2 to 0.3
Head circumference for age — z score			
Median	−0.1	0.0	−0.0
Interquartile range	−0.9 to 0.6	−0.7 to 0.6	−0.8 to 0.6
CDC class — no. (%)†			
N and A	237 (94.0)	121 (96.8)	358 (95.0)
B	11 (4.4)	3 (2.4)	14 (3.7)
CD4 percentage			
Median	35.1	35.6	35.2
Interquartile range	29.1 to 40.8	29.1 to 43.8	29.1 to 41.2
CD4 cell count — per mm^3			
Median	2035	2044	2039
Interquartile range	1519 to 2754	1585 to 2960	1541 to 2789

Variable	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)
Hemoglobin — g/dl			
Median	10.1	10.2	10.1
Interquartile range	9.3 to 10.7	9.4 to 11	9.4 to 10.8
Total lymphocyte count — per mm^3			
Median	6065	6250	6110
Interquartile range	4510 to 7735	4450 to 7500	4490 to 7690
Plasma HIV-1 RNA — log_{10} copies/ml			
Median	5.9	5.9	5.9
Interquartile range	5.6 to 5.9	5.6 to 5.9	5.6 to 5.9

[*] Information was missing for four infants in the early-therapy group and one infant in the deferred-therapy group.

[†] The Centers for Disease Control and Prevention (CDC) classification system is for HIV infection in children younger than 13 years of age.

Table 2

Mortality Rates.

Variable	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)	Hazard Ratio (95% CI)*	P Value
Deaths — no. (%)	10 (4)	20 (16)	30 (8)	0.24 (0.11–0.51)	<0.001
Person-years of follow-up — no.	205	94	299		
Overall death rate per 100 person-years — % (95% CI)	4 9 (2.3–9.0)	21.2 (13.0–32.7)	10.0 (6.8–14.3)		
Rate of death during follow-up — % (95% CI)					
0–13 wk	9.8 (3.6–21.4)	40.6 (21.0–71.0)			
>13–26 wk	3.7 (0.4–13.2)	19.7 (6.4–45.9)			
>26–52 wk	3.1 (0.4–11.2)	6.8 (0.8–24.7)			

*Hazard ratios for death are for the comparison of the early-therapy groups with the deferred-treatment group.

Table 3

Adverse Events.

Adverse Event	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)
All adverse events — no. of events	1356	1063	2419
Clinical adverse event			
Stage C event — no. of events			
HIV wasting	3	3	6
Pneumocystis jiroveci pneumonia	0	4	4
Recurrent bacterial infections	0	2	2
HIV encephalopathy	1	8	9
CMV pneumonitis	0	2	2
Disseminated CMV infection	0	1	1
Esophageal candidiasis	0	1	1
Extrapulmonary tuberculosis	1	1	2
Severe stage B event — no.			
Failure to thrive [*]	12	12	24
Lymphoid interstitial pneumonitis	0	1	1
Chronic lung disease	1	0	1
Stage C or severe stage B event			
No. of events	18	35	53
No. of participants with event [†]	16	32	
Most frequently reported infections — no. of infections (no./100 person-years)			
Grade 3 or 4 gastroenteritis	29 (14.1)	36 (38.2)	65 (21.7)
Grade 1 or 2 gastroenteritis	180 (87.5)	123 (130.5)	303 (101.0)
Grade 3 or 4 pneumonia	25 (12.2)	25 (26.5)	50 (16.7)
Grade 1 or 2 pneumonia	66 (32.1)	56 (59.4)	122 (40.7)
Meningitis	1 (0.5)	6 (6.4)	7 (2.3)
Tuberculosis	17 (8.3)	19 (20.2)	36 (12.0)
Laboratory grade 3 or 4 adverse event — total no. of events (no. of drug-related events) [‡]			
Alanine or aspartate aminotransferase elevation	6 (2)	1 (0)	7 (2)
Anemia	5 (3)	2 (1)	7 (4)
Neutropenia	19 (10)	3 (3)	22 (13)
Thrombocytopenia	3 (1)	1 (0)	4 (1)
Elevated γ-glutamyltransferase [§]	0 (0)	2 (0)	2 (0)
Hypernatremia	6 (0)	5 (0)	11 (0)
Hyponatremia	1 (0)	1 (0)	2 (0)
Hyperkalemia	1 (0)	0 (0)	1 (0)
Hypokalemia	1 (0)	1 (0)	2 (0)
Total events	42 (16)	16 (4)	58 (20)
Serious adverse event — no. (%)			

Adverse Event	Early Antiretroviral Therapy (N = 252)	Deferred Antiretroviral Therapy (N = 125)	Total (N = 377)
Participants with ≥1 event¶	55 (21.8)	55 (44.0)	110 (29 2)
Death	10 (4.0)	20 (16.0)	30 (8.0)
Life-threatening event	9 (3.6)	4 (3.2)	13 (3.4)
New or prolonged hospitalization	41 (16.3)	46 (36.8)	87 (23.1)
Persistent or significant disability or incapacity	0	1 (0.8)	1 (0.3)

* Failure to thrive was defined as the documented loss of body weight or body weight crossing two major percentile lines (50th, 25th, 10th, or 5th) and failure to gain weight within 4 weeks with standard management, with no other cause identified during investigation, or for patients already below the 5th percentile, failure of weight gain to parallel the 5th percentile (in the absence of chronic diarrhea and fever for >30 days).[13] CMV denotes cytomegalovirus.

† Some of these participants may have had more than one event.

‡ This category includes all adverse events deemed to be possibly, probably, or definitely drug-related.

§ An elevation was considered to be present if the assayed value was 10 or more times the upper limit of the normal age-adjusted range.

¶ A participant may have had an event that was included in more than one category of serious adverse events.

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Structural organization of authentic, mature HIV-1 virions and cores

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Mature, infectious HIV-1 particles contain a characteristic cone-shaped core that encases the viral RNA and replication proteins. The architectures of mature virions and isolated cores were studied using cryo-electron microscopy. The average size (~145 nm) of the virion was unchanged during maturation. Most virions contained a single core but roughly one-third contained two or more cores. Consideration of the capsid protein concentration during core assembly indicated that core formation *in vivo* is template-mediated rather than concentration-driven. Although most cores were conical, 7% were tubular. These displayed a stacked-disc arrangement with 7-, 8-, 9- or 10-fold axial symmetry. Layer line filtration of these images showed that the capsid subunit arrangement is consistent with a 9.6 nm hexamer resembling that previously seen in the helical tubes assembled from purified capsid protein. A common reflection (1/3.2 nm) shared between the tubular and conical cores suggested they share a similar organization. The extraordinary flexibility observed in the assembly of the mature core appears to be well suited to accommodating variation and hence there may be no single structure for the infectious virion.

Keywords: capsid protein/cryo-electron microscopy/fullerenes/macromolecular assembly/retrovirus

Introduction

Formation of mature, infectious HIV-1 proceeds through two steps: budding of the immature, non-infectious particle and maturation to the infectious virion (for reviews, see Vogt, 1997; Freed, 1998; Goettlinger, 2001). Particle formation requires the transport of viral Gag polyproteins to the plasma membrane where they associate with other viral and cellular components to produce a budding structure. Virions are released from the cell surface as immature, non-infectious particles containing a spherical layer of Gag polyproteins underneath the viral membrane. The action of the viral protease (PR) on these polyproteins initiates virus maturation concomitant with or after budding (Vogt, 1997). Proteolytic cleavage at defined sites, in a defined order, leads to the formation of the proteins matrix (MA), capsid (CA), nucleocapsid (NC), p6 and two smaller peptides. Proteolytic processing is not required for particle formation or budding. It is required for condensation of the immature, spherical Gag shell to the mature, mostly cone-shaped, core and for the development of HIV-1 infectivity. Prevention of cleavage and maturation using specific inhibitors of the viral PR (Ashorn *et al*., 1990) is a mainstay of current therapeutic regimens for AIDS patients (Sepkowitz, 2001).

Assembly and budding of immature retroviral particles requires only the viral Gag polyproteins (Coffin *et al*., 1997; Vogt, 1997), which assemble at the plasma membrane or in the cytosol, depending on the specific virus. Most likely, assembly is predominantly driven by protein protein interactions of the C-terminal domains of CA within the Gag polyprotein and is enhanced by membrane association through the N-terminal MA-domain and by RNA protein interactions mediated by the NC-domain. This leads to three concentric layers of interaction in the Gag shell, yielding a stable protein coat that must be destabilized to prepare for disassembly in the newly infected cell. To achieve this, the Gag shell of the immature particle is dissociated during maturation. Condensation of the inner core structure should therefore be considered as a second assembly step, occurring at millimolar protein concentration in the confined space of the extracellular virion. Protein protein interactions of CA molecules are likely to promote the formation of the mature CA shell, but the driving force for core formation remains unclear. The process could be driven by the concentration of the CA protein, comparable to the *in vitro* assembly of CA that generates helical tubes. Alternatively, it could be promoted by interaction with the ribonucleoprotein complex (RNP) or another pre-existing structure that would serve as a template for core formation, akin to epitaxial growth (Bergfors, 1999). The template-mediated process would lower the critical concentration for core formation so that the number of cores would reflect the number of templates rather than the CA concentration.

The morphology and architecture of immature and mature HIV-1 particles and other retroviruses has been studied for many years (reviewed in Vogt, 1997). Structural analysis of the immature particle has been facilitated through the expression of Gag polyproteins or domains in tissue culture, to form virus-like particles (VLPs) that share many features with the immature virion. Assembly of spherical particles closely resembling the immature CA shell was even possible in a completely *in vitro* system using bacterially expressed HIV (Gross *et al*., 2000) or Rous sarcoma virus (Campbell and Vogt,

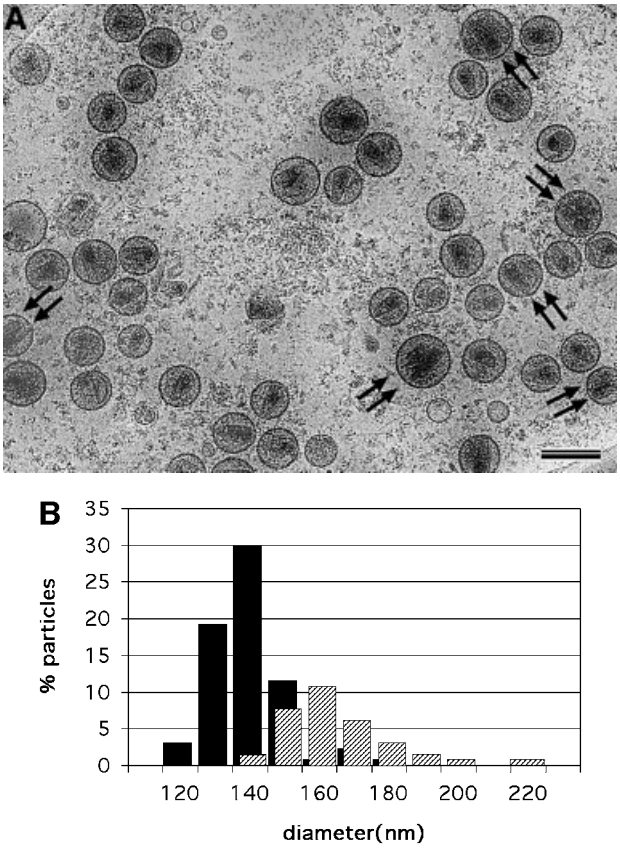

Fig. 1. (**A**) cEM of mature HIV 1. Mature HIV 1 particles were harvested from infected MT 4 cells before a cytopathic effect was established. Virions were inactivated with paraformaldehyde before vitrification. Released virus particles displayed a broad range of diameters, extending from 120 to 200 nm (mean 145 ± 25 nm, *n* 255). The majority of virions contained a single core, but a significant num ber of particles (32.6%) contained two or more cores. Virions with a single core were clearly smaller in diameter than virions with two cores (marked with double arrows). Scale bar represents 150 nm. (**B**) The distribution of sizes of HIV 1 virions bearing one and two cores. The distribution of radii is displayed for 132 virions. Particles that appeared to contain more than two cores were excluded. The mean size of the virions containing a single core (134 ± 11 nm, *n* 89, black bars) was significantly smaller (for equal means, t 9.3, Prob. 1.2×10^{15}) than the mean size of virions containing two cores (158 ± 16 nm, *n* 43, hashed bars).

1997; Yu *et al*., 2001) Gag-derived proteins. Work on immature particles by negative staining and freeze substitution (Grief *et al*., 1994; Nermut *et al*., 1994, 2002; Kakker *et al*., 1999) suggested that the proteins underlying the surface adopt a hexagonal net. Similar nets have been described in two-dimensional crystals of lipid-linked retrovirus capsid proteins (Barklis *et al*., 1996, 1998; McDermott *et al*., 2000; Mayo *et al*., 2002a,b). Detailed analysis of HIV-1 Gag-VLPs and of authentic immature retroviral particles by cryo-electron microscopy (cEM) revealed that particles are heterogeneous in size and shape and exhibit no overall (e.g. icosahedral) symmetry (Fuller *et al*., 1997; Yeager *et al*., 1998; Wilk *et al*., 2001), but show areas of local symmetry (Fuller *et al*., 1997; Wilk *et al*., 2001). The architecture of the immature virus appears relatively simple: the uncleaved Gag polyproteins are arranged radially with the MA domains at the membrane and the C-terminal ends pointing inward (membrane-MA-CA-NC-p6) (Wilk *et al*., 1999). This radial sequence is maintained in the mature virion. A layer of MA proteins remains beneath the membrane, while the processed CA proteins form the shell of the mature core (Gelderblom *et al*., 1987). The RNP including the viral replication proteins, forms within this core by condensation of the genome with NC.

The biochemical and structural characterization of the morphologically defined mature HIV-1 core has been difficult. Removing the envelope of the mature virion by detergent treatment led to rapid disintegration of the inner structures. Recently developed protocols have allowed preparation of intact cores for negative-stain EM and biochemical analysis (Kotov *et al*., 1999; Accola *et al*., 2000; Welker *et al*., 2000). These studies provided an overview of the core geometry, documenting its variability and the approximate ratio of its constituents (Welker *et al*., 2000). Studies on *in vitro* assembled particles have shed additional light on core formation. Retroviral CA as well as longer proteins containing the NC domain (e.g. CA-NC) assemble *in vitro* into helical tubes with diameters between 300 and 800 Å and lengths of up to several microns (Ehrlich *et al*., 1992; Campbell and Vogt, 1995, 1997; Gross *et al*., 1997; Ganser *et al*., 1999). Helical reconstruction of HIV-1 CA tubes (Li *et al*., 2000) from several helical families showed that they are formed of hexameric rings of CA protein. The hexamer displayed an exterior diameter of ~100 Å surrounding a central hole of ~25 Å diameter. The radial arrangement of the CA protein was similar to that in the immature virion. The N-terminal CA domain was located on the outside and the observed ~72 Å thickness corresponded to the long axis of the CA subunit in Gag.

Purified HIV-1 CA protein can also assemble to form conical structures that are reminiscent of the authentic HIV core (Ganser *et al*., 1999), suggesting that the *in vitro* assemblies are relevant to the cores found in the virus, but the structure and formation of the core within the authentic virion has not been analyzed to date. Ganser *et al*. (1999) proposed that the core maintained local 6-fold symmetry by forming a fullerene cone. A hexagonal net can be closed by the inclusion of 12 pentagons. These are equally spaced to close the net in an icosahedron (Caspar and Klug, 1962). When the pentagons are arrayed so that six are at either end of an elongated structure, a tube results. An uneven distribution of pentamers, such as five at one end and seven at the other, results in a closed cone-like structure. This is well described by Ganser *et al*. (1999) and the reader is directed to this paper for a complete explanation and related references.

Here, we present a cEM analysis of mature HIV-1 particles obtained from infected T cells and of virion-derived cores. These studies extend the *in vitro* experiments by addressing two issues. The first is the relevance of the helical tubes to the architecture of authentic HIV cores. We show that the basic unit of the authentic, mature core is consistent with a hexamer that appears similar to the one forming the *in vitro* helical tubes. The second is the effect of the conditions of core formation in the virion. These are quite distinct from those used for *in vitro* assembly studies. The frequent observation of multiple cores within single virions, combined with the calculation of the CA protein concentrations during core formation,

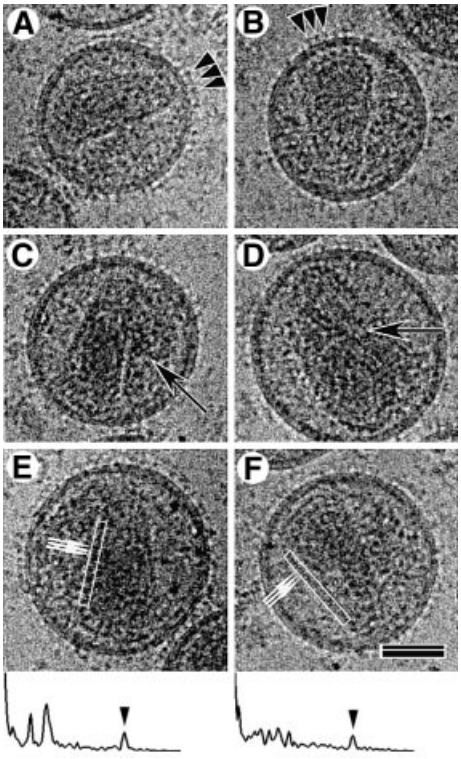

Fig. 2. cEM analysis of the mature virion. Most mature virions dis played regular membrane projections (**A** and **B**, arrowheads) and con tained cone shaped cores. Many particles contained a complete second core, which can be conical, tubular (**C** and **D**, arrows) or amorphous. cEM allowed the visualization of the regular arrangement of core sub units (small arrows in **E** and **F**), which were spaced at 3.2 ± 0.48 nm, *n* 60. The power spectra of the boxed regions demonstrating the pres ence of the 3.2 nm repeat (arrowheads) along one side of the cone are shown below panels (E) and (F). The broad end of the conical core was usually located in proximity to the membrane; however, no connection could be observed. The scale bar represents 50 nm.

suggests that the assembly process is template-mediated rather than concentration-driven as it is *in vitro*.

Results

The organization of mature HIV-1

We obtained a concentrated, high-titer (~10^9 infectious units per ml) HIV-1 preparation containing minimal cellular contaminants by combining a high-multiplicity infection with harvesting at early time points and rapid purification of virus (Welker *et al*., 2000). SDS PAGE analysis of the concentrated preparation showed that the Gag proteins were the main constituents and there was minimal contamination with cellular proteins (data not shown). Virus particles were pelleted through a cushion of sucrose and vitrified immediately as described previously (Fuller *et al*., 1997). Rapid sample preparation minimized deleterious effects on the particle structure. Typical samples showed a large number of mature HIV-1 particles and lacked large, membranous, cellular contaminants (Figure 1A). The diameter of the mature particles (145 ± 25 nm, *n* = 255) was very similar to that previously reported for immature particles (139 ± 16 nm, *n* = 197) (Wilk *et al*., 2001). The interiors of most particles contained a central, dense core structure. Some density was observed within the virion outside the core. No structures that could correspond to the previously reported lateral bodies (Gelderblom *et al*., 1987) were visible. Immature virions were rarely observed and represented <1% of the preparation.

Images of vitrified mature HIV-1 showed closely packed patches of surface projections, extending ~7.5 nm from the virion surface (arrowheads in Figure 2A and B). Analysis of the density distribution within the lipid bilayer (data not shown) indicated that it had the same three-layered appearance as observed for immature HIV-1 (Wilk *et al*., 2001) and the VLPs resulting from Gag expression (Fuller *et al*., 1997). The majority of the enveloped virus particles in the preparation contained a single core structure. Surprisingly, a significant fraction of the mature particles contained a second core assembly (32.6%, *n* = 132) (Figure 1A, double arrows; Figure 2C and D, arrows). Particles containing three distinct cores were observed very rarely. Most cores exhibited a conical shape but a small fraction showed a tubular or amorphous morphology. Mature HIV-1 particles containing two cores had a significantly larger particle diameter (159 ± 16 nm) than particles with only one core (134 ± 11 nm) (Figure 1B). When two or more cores were observed they appeared to be roughly the same size as the cores in virions that contained only one, although overlap of the two cores made it difficult to determine this precisely.

Closer examination of the images revealed that the ends of the conical cores were often located near the viral membrane (Figure 2). A gap was usually observed between the inner leaflet of the membrane and the core. This makes the presence of a solid physical link (Gelderblom, 1991) between the membrane and the viral core unlikely. A regular arrangement (3.2 ± 0.5 nm, *n* = 60) of subunits could often be seen along one edge of assembled cores (Figure 2E and F, arrows and power spectra of boxed regions). Such a repeating feature gives rise to a diffraction peak in the power spectrum of the image (see below).

Morphology of core particles isolated from mature HIV-1

HIV-1 preparations produced as described above were lysed with detergent to isolate cores. After treatment with 0.5% Triton X-100 for 2 min, cores were collected by a brief spin in a microcentrifuge as described previously (Welker *et al*., 2000). Analysis of the core preparation by cEM revealed a large number of intact cores lacking the viral membrane (Figure 3). Isolated cores were heterogeneous in size and shape with a morphology similar to that observed in complete virions. The preparation contained predominantly cone-shaped core structures of various sizes, as well as a minor fraction of tubular (Figure 3, arrows) and polymorphic shapes. Many cores exhibited a region of increased density at the broad end of the cone (Figure 3, arrowheads), which is likely to correspond to the viral RNP.

Measurements of the overall length, diameter and the cone angle at the narrow end were performed for 267 conical cores. Cores were excluded if they appeared to be damaged or acutely tilted so that all three parameters could not be measured reliably. As shown in Figure 4, the three parameters showed nearly Gaussian distributions with a

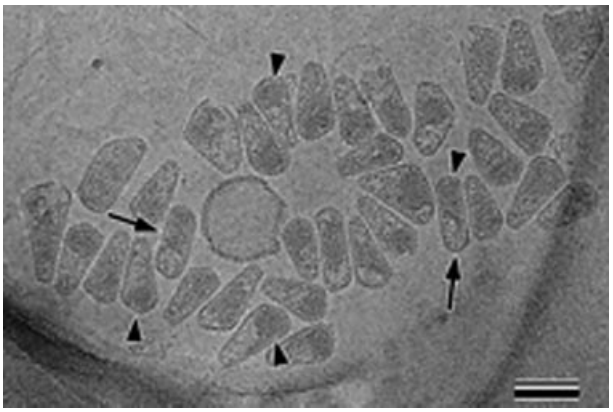

Fig. 3. Overview of isolated HIV 1 cores in cEM. Intact cores were prepared by brief detergent treatment and centrifugation in a microcentrifuge as described (Welker *et al*., 2000). This figure shows a representative cEM image depicting isolated cores of heterogeneous size and shape. The majority of the isolated cores displayed a conical shape while a minor fraction was found to be tubular (~7%, arrows). In most cases, the regular arrangement of the wall subunits was preserved. Many cores showed an increased density at the broad end, presumably due to the condensed viral RNP in this position (arrowheads). The scale bar represents 100 nm.

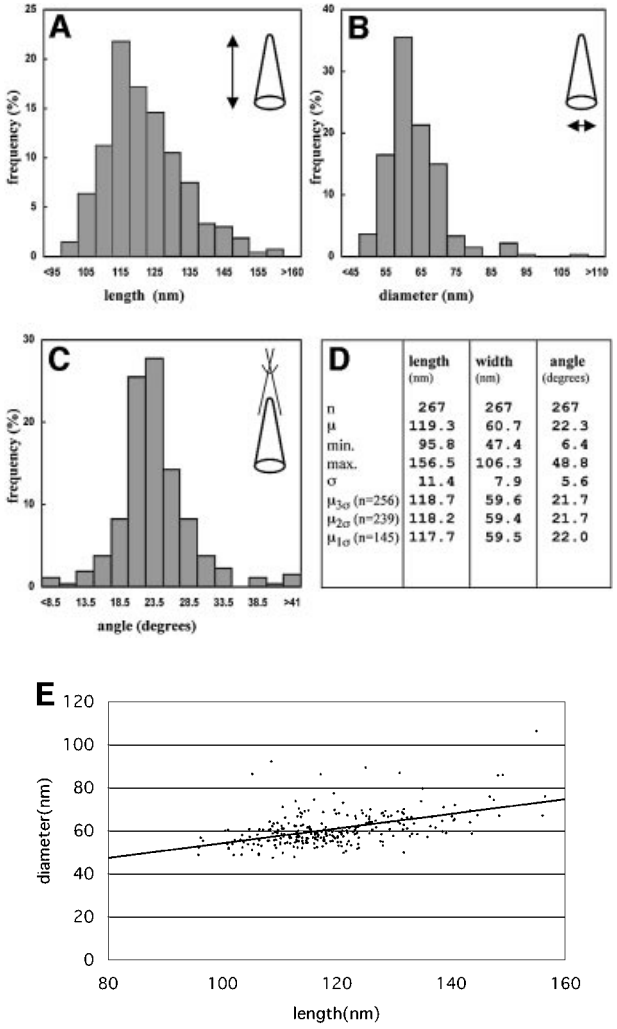

Fig. 4. Size and geometric variation of HIV 1 cores. The histograms show the distribution of length (**A**), diameter (**B**) and angle at the narrow end (**C**) of conical HIV 1 cores. Length and diameter were measured once for each core, while the mean of three independent measurements was used in case of the angle. Particles were excluded if the standard deviation of three measurements for the cone angle was >15% of the respective mean, or if all three parameters could not be measured. Arbitrary size classes were defined for each parameter, and the frequency of cores found for each class was plotted. (**D**) A tabulated version of the results. Abbreviations: *n*, number; μ, mean; min., minimal value; max., maximal value; σ, standard deviation; $\mu_{3\sigma}$, $\mu_{2\sigma}$ or $\mu_{1\sigma}$, mean calculated from the fraction of particles within the range of three (n 256), two (n 239) or one (n 145) standard deviation of the overall mean, respectively. The correlation between the width at the broad end of the core and the overall length is shown in (**E**). The slope of the line corresponded to an included angle of 19.7 ± 2°.

slight positive skew. The average particle length (119 ± 11 nm) was measured along the central core axis and the maximum diameter (60 ± 8 nm) was measured perpendicular to this axis (Figure 4A, B and D). Measurement of the angle at the tip of the core gave 22 ± 6° (Figure 4B), and was associated with a larger degree of variation than measurements of length and diameter (Figure 4D). The potential impact of a few aberrant cores was evaluated by recalculating the means using only the particles within the 3σ, 2σ or 1σ ranges. The resultant means ($\mu_{3\sigma}$, $\mu_{2\sigma}$ and $\mu_{1\sigma}$ in Figure 4D) varied only marginally (<2.7%) from those calculated for the whole data set. Hence the variance in the values represents the true variation in the population rather than the contribution of a few outliers.

We reasoned that the effect of tilt on apparent cone angle should appear in a comparison of long cores (which must lie relatively parallel to the water layer) with short ones (which can adopt a more acute angle within the layer). Plotting the width of the broad end of the core versus its length (Figure 4E) yielded a correlation coefficient of 0.35 ± 0.04, corresponding to an included angle of 19.7 ± 2.1° [$2\tan^{-1}(0.35/2.0)$]. This provided further evidence for the overall conical shape of the core. We observed no correlation between this derived cone angle and length (Figure 4E). Hence, tilt did not significantly affect the apparent cone angle to within the precision of these measurements. Any possible effect of tilt on the measurements would be small; a consistent 10° tilt in the cores would only increase the apparent cone angle by ~1°.

The structural organization of authentic, mature HIV-1 cores

The removal of the viral membrane allowed us to examine the ends of the isolated cores closely. Cores appeared to be closed structures. Examination of individual cores in single (Figure 5) and stereo images (data not shown) revealed continuous density along the ends rather than the broken density that would indicate a hole. The appearance of conical cores was complex because of the superposition of the two sides of the structure seen in the cEM image. The 3.2 nm spacing between repeating units was most easily seen along the long edges of the core (Figure 5C, boxed region, and D), as it was in the intact virions. Repeating units were never clearly seen on both edges of the same core as expected for a fullerene cone (see below and Li *et al*., 2000).

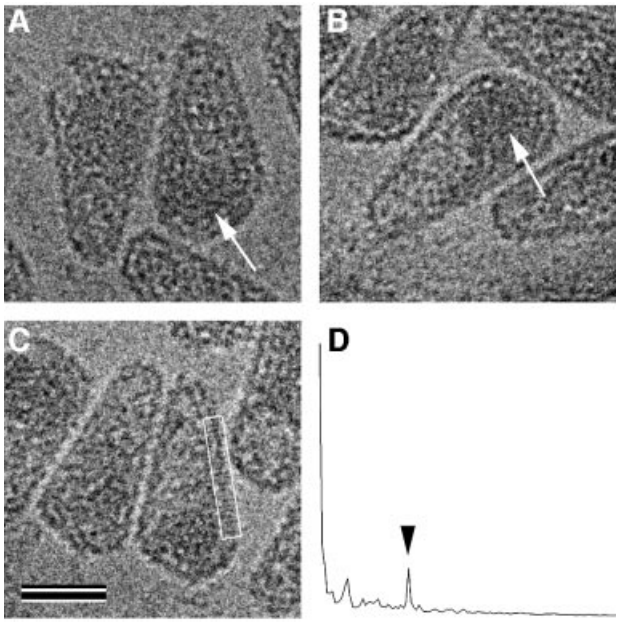

Fig. 5. Isolated HIV 1 cores retain well ordered subunits and terminal structures on both ends. (**A**–**C**) Typical cores appeared closed at both ends. The white arrows mark the positions of density that we presume to correspond to the RNP in the cores. The scale bar represents 50 nm. The power spectrum of the region boxed in (C) is shown in (**D**). The detergent treatment had little effect on the regular arrangement of CA subunits, as indicated by the preservation of the ~3.2 nm reflection (arrowhead).

The packing of subunits in the conical cores was difficult to analyze. The classical tools of Fourier analysis that are appropriate for crystalline or helical structures exploit the fact that the substructure repeats with a defined distance and hence gives rise to diffraction maxima in a power spectrum. An arrangement of subunits that generates a conical structure does not repeat with distance. The power spectrum of such an object will not display, for example, the sharp layer lines of a helical object. The cores that displayed a tubular morphology (~7% of the total, 18 of 267) were more tractable (Figure 6A) since they did display a simple repeating pattern as evidenced by the lines comprising the power spectra (Figure 6B). We therefore focused on these tubular cores for analysis.

Nineteen of 25 apparently tubular cores analyzed exhibited peaks in their power spectra. Eighteen of these cores showed similar power spectra and were analyzed further. Tubular cores had an average diameter of 44.3 ± 4.3 nm. Their lengths (119.7 ± 20.1 nm) were similar to the length of conical cores. Figure 6B shows the computed power spectrum of a tubular core (raw image in Figure 6A) that displayed peaks corresponding to the repeated distances in the structure. These layer lines were simple multiples of ~9.6 nm (9.6 ± 0.3 nm). Distinct classes of tubular cores could be distinguished by their diameters. The observed diameters were multiples of 5.4 ± 0.2 nm (N = 7, 8, 9, or 10 with R = 0.96). The positions of the diffraction maxima (Figure 6C; Supplementary data available at *The EMBO Journal* Online) did not vary with core diameter as shown in Figure 6G, in which tube diameter was plotted against the repeat distances for the tubular cores analyzed in this study. This differs from the case with classical helical symmetry, such as the *in vitro*-assembled CA tubes (Li *et al*., 2000), in which the spacing of the layer lines varies with the tube diameter.

Image processing revealed the structure of the repeating unit. Correcting the contrast transfer function (Erickson and Klug, 1971; Mancini and Fuller, 2000; Mancini *et al*., 2000) on the individual images and summing the aligned defocus pairs prior to filtering enhanced the contrast. These operations increased the strength of the layer lines but did not affect the symmetry of the resulting image. The fact that information about the repeating unit is found on the layer lines allows one to filter the transform and back transform to show the repeating unit. Selecting the strongest layer lines (which are multiples of 1/9.6 nm) (Figure 6E) and zeroing the transform between them generated a filtered image of the repeating unit (Figure 6D). A 2-fold symmetry is evident in Figure 6D (2-fold phase residual 34°, where a residual of 0° would correspond to perfect 2-fold symmetry and 90° to random). Application of this 2-fold symmetry to the filtered image yielded the projection shown in Figure 6F for this N = 8 tubular core. The filtered, averaged image showed a hexameric unit with a diameter of ~9.6 nm. The clarity of the hexamers in Figure 6 reflects the fortuitous occurrence of a tube that is almost directly perpendicular to the direction of view and in the proper axial rotation. In this tube, the hexamers on the top and bottom superimposed. A small tilt would have generated a projection in which the hexamers in the body of the tube interfere and produced a less interpretable image. Our conclusion that hexamers comprise the tubes rests on the conservation of the repeat distance and the quantized distribution of diameters that matched that expected for a hexagonal array. Figure 7 is an illustration of this arrangement that is consistent with the statistical analysis of the tube geometry.

Discussion

A detailed understanding of the formation of infectious HIV-1 particles requires structural information regarding the mature virion, its assembly intermediates and the relevant building blocks. Our current knowledge regarding the structure and organization of authentic mature HIV-1 virions and cores is mainly derived from conventional EM. Here, we report a cEM study of mature, infectious HIV-1 particles that extends our previous work on immature virions (Wilk *et al*., 2001) in order to characterize the authentic virion. These studies document the intrinsic variability of HIV-1 particle sizes and shapes and suggest the consequent robustness of the assembly process. HIV-1 assembly does not follow an exact blueprint for the production of identical particles but rather obeys rules that result in the production of variable particles.

The intact mature virion

Intact mature HIV-1 particles displayed projecting spikes on the surface, which are likely to correspond to the viral glycoproteins. Our previous analysis of immature HIV-1 particles revealed a comparable density of surface projections (Wilk *et al*., 2001). The lipid bilayer itself displayed the same overall organization as reported for immature HIV-1. The dense layer of protein located just below the

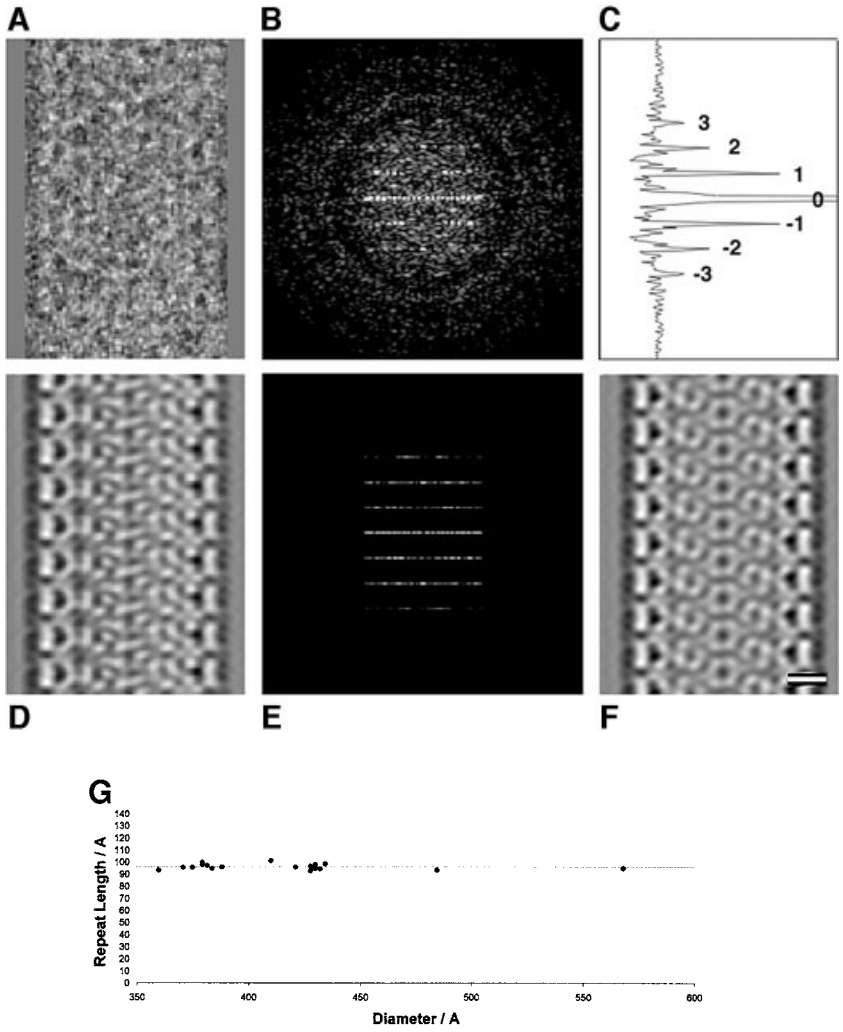

Fig. 6. Image processing of a tubular core. The steps of image processing are shown for a tubular core. (**A**) The raw image of the isolated core. The power spectrum of this core is shown in (**B**), with the corresponding collapsed intensity profile in (**C**). The orders of the 1/9.6 nm reflections were labeled. Suppression of the off layer line noise in (B) gives the filtered power spectrum in (**E**). The corresponding layer line filtered image is shown in (**D**). (**F**) The image in (D) after application of 2 fold symmetry. (**G**) The repeat length of the tubular cores is independent of their diameter. The scale bar in (F) represents 10.0 nm.

membrane and attributed to the MA domain of Gag in the immature particle (Fuller *et al*., 1997; Wilk *et al*., 2001) was maintained in the mature particle. Comparison of the average sizes of authentic mature and immature HIV-1 particles revealed that maturation occurs without a significant change in diameter.

Architecture of isolated cores

cEM analysis confirmed that the variation in size and shape previously observed for negatively stained cores (Welker *et al*., 2000) was an intrinsic property of the core rather than a reflection of dehydration or preparation for EM. Similar results were recently reported for Rous sarcoma virus (Kingston *et al*., 2001; Yu *et al*., 2001). cEM yielded larger values for the length (119.3 nm) and width (60.7 nm) of isolated cores in comparison with the values measured by negative staining (102.9 and 52.4 nm, respectively (Welker *et al*., 2000). This presumably reflects the lack of shrinkage in the hydrated samples. *In vitro*-assembled cores varied in length between 100 and 300 nm (Ganser *et al*., 1999). Comparison of the cEM images of cores within mature virus with those of the isolated cores revealed that both display the characteristic 3.2 nm edge repeat, indicating that the isolation procedure did not significantly distort the core.

Ganser *et al*. (1999) presented an elegant fullerene cone model for the HIV core based on the structure that their group determined for *in vitro*-assembled helical tubes of CA protein (Li *et al*., 2000). The fullerene model predicts that core assemblies will adopt one of a set of defined geometries. These geometries permit the preservation of hexagonal (p6) symmetry in the lattice with 12 pentameric vertices allowing the curvature of the structure to produce a closed core. Ganser *et al*. (1999) showed that CA-NC assembles *in vitro* with RNA to adopt a set of included cone angles (19.2, 38.9, 60, 83.6 and 112.9°) corresponding to the defined set of fullerene cone geometries (Ge and Sattler, 1994). The 19.2° species (corresponding to five

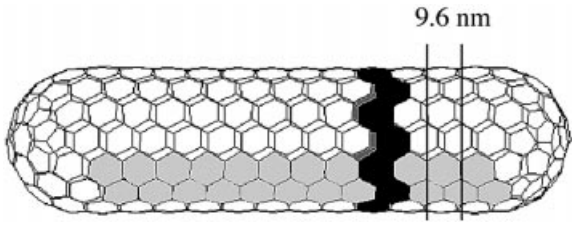

Fig. 7. Schematic of a stacked disc fullerene tube. The figure illustrates one possible structure of a stacked disc fullerene tube. One disc in the stack is shaded black. Removing or adding a strip of hexamers such as that shaded in gray varies the width of the tube body. The tube shown is formed from eight such strips. The tube ends illustrated represent one of a number of possible constructions. The 9.6 nm repeat is marked.

pentamers at the narrow end and seven at the wide end) formed the majority of the population. The authors suggested that authentic HIV-1 cores should follow the same distribution of angles. Our Fourier filtered images of tubular cores (Figure 6) revealed a hexagonal unit in authentic HIV-1 cores. The size of this hexamer was similar to that seen by Li *et al*. (2000), providing direct evidence that the hexameric unit seen in *in vitro*-assembled CA tubes is indeed relevant to the authentic core. The fact that the tubular cores shared the 3.2 nm spacing with the conical ones suggests that the local packing of the hexamers was similar in both structures. The observed variation in the size and end shape of the conical cores can be accomodated in the fullerene cone model by variation of the number of hexamers and the precise positioning of the pentameric defects. The average angle of the authentic cores (22 ± 5°) was very similar to the 19.2° predicted by fullerene cone geometry. The authentic conical cores appeared to be more restricted in their included angle than the *in vitro*-assembled ones, since only the 19.2° cones were observed. This may reflect biological control of core assembly.

The filtered, symmetrized image of a tubular core (Figure 6F) could be interpreted as layers of hexamers. The hexamers in alternate layers were staggered relative to each other so that the layers were paired. The paired layers formed discs that repeated with a constant spacing of 9.6 nm. This arrangement of hexameric units is illustrated in Figure 7. It corresponds to a degenerate case of helical symmetry called a 'stacked disc'. Such an arrangement has been observed previously in several biological contexts, most notably as an intermediate in tobacco mosaic virus assembly (Butler and Klug, 1971). The particular arrangement of stacked discs has also been described for fullerene tubes, where it is denoted the 'armchair' form because the hexamer edges are perpendicular to the long axis of the tube (Yacobsen and Smalley, 1997).

The observed 9.6 nm reflection is caused by the spacing of the hexamers (marked in Figure 7). The discrete variation in tube diameter would correspond to the removal or addition of pairs of strips of hexamers (7, 8, 9 or 10) (Figure 7) along the length of the tube. This would alter the circumference by multiples of 16.6 nm and change the diameter by multiples of 5.3 nm, which is indistinguishable from the observed 5.4 ± 0.2 nm. The 3.2 nm reflection must result from features within the hexamer such as domains of the individual proteins coinciding with the third order of the 9.6 nm repeat. The packing in the tubular cores provides some insight into the arrangement in the conical ones. The edges of the conical cores showed a reflection of 3.2 nm (3.2 ± 0.5 nm, $n = 60$). The tubular cores also showed a 3.2 nm reflection. Hence, local packing of the hexamers was similar in the two structures although the overall arrangement that results in the formation of the tubular core must differ from the one that results in a conical core. A more complete determination of the structure of the conical cores will require the use of methods, such as tomography, that do not require averaging of this variable structure.

Formation of multiple cores: implications for the mode of assembly

The presence of HIV-1 particles with multiple cores has been noted previously (e.g. Gelderblom, 1991); however, they were seen as rare events. Previous analyses of HIV-1 particles used primarily thin-section electron microscopy, which will only detect multiple cores if they both lie within the plane of the section and present a recognizable profile. Indeed, the fact that multiple cores have been observed by conventional methods at all suggests that they are not rare events. Our cEM analysis of mature HIV-1 preparations showed that roughly one third (32.6%) of the mature particles contained two discrete core structures. Multiple cores were also common in other preparations of HIV-1 and of HIV-2 (J.A.G.Briggs, J.Daeke, H.-G. Kräusslich and S.D.Fuller, manuscript in preparation).

Virions containing two cores were larger on average than those containing a single core. This observation has implications for the mechanism of core formation. Two fundamentally different mechanisms can be distinguished. Core formation could be driven by the concentration of CA protein, as it is during assembly of helical tubes *in vitro*. Multiple cores would form when the CA concentration exceeds the threshold required for assembly. Alternatively, a template, such as the RNP, could initiate core formation. Multiple cores would form in a virion when it contained multiple templates. Our measurements of the virion diameters led to a quantitative picture of core formation that distinguishes these possibilities.

Recent scanning transmission EM measurements of the similarly sized Mason Pfizer monkey virus (1900 2100 molecules; Parker *et al*., 2001), allows one to estimate that the average HIV-1 particle contains ~2100 Gag monomers. These values would correspond to a CA concentration of 3.5 mM (1.8 mM dimers since Gag and CA are believed to be dimeric; Gamble *et al*., 1997; Scarlata *et al*., 1998; Worthylake *et al*., 1999). Provided that the Gag Gag spacing in small and large particles is the same, one can predict that the group of larger particles (159 nm diameter; Figure 1B) that contained two cores will have a lower CA concentration (1.5 mM dimers) than the group of smaller (134 nm) particles with a single core (1.8 mM) because the ratio of surface area to volume decreases with radius.

The number of CA molecules consumed by the formation of a cone can be estimated from the packing density in the helical assemblies (Li *et al*., 2000) and from the measurements of cone angle, length and maximum width, giving 625 dimers when the effect of the end

structures is considered. The tubular cores described here should contain ~550 dimers. Formation of the first core therefore leaves concentrations of 0.70 and 0.72 mM CA dimers in the small and large particles, respectively, which is not sufficiently different to explain formation of a second core only in the latter case. At no point before or after formation of a single core is the CA concentration in the larger double-cored particles higher than that in the smaller single-cored particles and hence concentration cannot drive initiation of formation of multiple cores. Taken together, these considerations suggest that core formation during HIV-1 maturation is template-mediated. This conclusion is strengthened by recent scanning transmission EM measurements, which suggest that our current estimate of the total amount of CA protein in a virion may be too low (J.A.G.Briggs, M.C.Johnson, M.Simon, I.G.Gross, H.-G.Kräusslich, V.M.Vogt and S.D.Fuller, manuscript in preparation).

Why would larger particles be more likely to contain multiple templates? Possible templates include the RNP-complex, factors at the site of membrane closure upon virion release, and protein complexes formed upon initiation of polyprotein processing (e.g. CA pentamers). However, the latter two would not explain easily why particles containing two templates are larger on average. The domain model for immature particle formation (Fuller *et al*., 1997) suggests that larger particles would be formed by the accretion of smaller, RNA-containing domains. Immature particles consisting of two half-shells of Gag as well as confluent viral buds have been observed by thin-section EM analysis (Gelderblom *et al*., 1987, Gelderblom, 1991; Vogt, 1997). Incorporation of multiple RNAs could lead to formation of separate RNPs and hence separate templates. The hypothesis of RNP-template nucleated CA condensation would be consistent with the organization of assembly domains and the order of cleavages. The C-terminal region of CA, the adjacent spacer peptide and NC appear to form a continuous assembly domain, essential for formation of the immature virion, and also for packaging the RNA genome. Release of the RNA-associated NC is a first step in Gag polyprotein cleavage, which destroys this assembly domain, separates CA from NC and allows condensation of the RNP that could subsequently function as a template for CA condensation. If multiple cores are indeed due to the presence of multiple RNP templates, one would predict that a significant number of virions contain four copies of RNA (two RNA dimers). If infectious, such tetraploid virions could increase the potential for recombination.

Materials and methods

Cell culture and virus preparation

High titer virus preparations were produced as described previously (Welker *et al*., 2000). Briefly, MT 4 cells were maintained at 37°C, 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM glutamine and 5 mM HEPES. Stocks of HIV 1 strain NL4 3 (Adachi *et al*., 1986) were produced by transfection of HeLa cells. MT 4 cells were initially infected with cell free virus, and infected cultures were subsequently expanded by co cultivation. Virus preparations for isolation of HIV 1 cores were harvested before pronounced cytopathic effects were observed (24 32 h post infection). Virus containing supernatants were cleared by low speed centrifugation, filtered through 0.45 µm pore size cellulose acetate filters (Schleicher and Schuell) and analyzed for antigen content. Infectious titers of virus preparations were at least 2×10^7 $TCID_{50}$ per ml (tissue culture infectious dose 50% per ml) as determined by endpoint titration (Welker *et al*., 2000). Virus particles were concentrated from cleared culture medium by centrifugation through a cushion of 20% (w/w) sucrose in phosphate buffered saline (PBS) at 130 000 *g* for 2 h at 4°C. The pellet was slowly resuspended in PBS (~3.5 µl/ml initial culture volume). Prior to vitrification, intact HIV 1 particles were inactivated with 2% paraformaldehyde in buffer for 30 min on ice.

Isolation of HIV-1 cores

For isolation of virus cores (Welker *et al*., 2000), 40 µl of fresh virus suspension were mixed with an equal volume of 200 mM NaCl, 100 mM MOPS pH 7.0, and virions were lysed for 2 min at room temperature by adding Triton X 100 to a final concentration of 0.5%. HIV 1 cores were recovered by centrifugation in a microcentrifuge at full speed (13 800 *g*) for 8 min at 4°C. The pellets were washed twice with 100 mM NaCl, 50 mM MOPS pH 7.0, and resuspended in 8 µl of the same buffer. Core suspensions were processed immediately for further analysis without fixation.

Microscopy and image analysis

cEM was performed as described previously using a Philips CM200FEG operated at 200 kV at a magnification of 38 000× (Fuller *et al*., 1997; Wilk *et al*., 2001). Micrographs were digitized on a Zeiss SCAI scanner (Oberkochen, Germany) at a step size of 14 µm or a UMAX 3000 scanner at 8 µm step size. This is equivalent to 2.19 Å/pixel for the images analyzed for the reconstruction in Figure 6. Measurements were performed using the SPIDER image analysis program (Frank *et al*., 1996). The particle size was measured from the outer leaflet of the membrane and thus excludes the contribution of the viral glycoproteins. The defocus was determined from the positions of the local minima in a radially averaged power spectrum of the micrographs. We divided the transform of the image with the appropriate phase contrast transfer function (CTF) to correct its effect as described previously (de Haas *et al*., 1999; Mancini and Fuller, 2000; Mancini *et al*., 2000). Images were aligned by cross correlation after CTF correction and then layer line filtered and 2 fold averaged to produce the final projection. The 2 fold phase residual was calculated by cross correlating the 2 fold rotated image with the original.

Measurements of length, diameter and angle of isolated cores were performed as described previously (Welker *et al*., 2000) using the SigmaScan software package (Jandel Scientific). Statistical analysis was performed using Microsoft Excel.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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ARTICLES

Architecture and secondary structure of an entire HIV-1 RNA genome

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Single-stranded RNA viruses encompass broad classes of infectious agents and cause the common cold, cancer, AIDS and other serious health threats. Viral replication is regulated at many levels, including the use of conserved genomic RNA structures. Most potential regulatory elements in viral RNA genomes are uncharacterized. Here we report the structure of an entire HIV-1 genome at single nucleotide resolution using SHAPE, a high-throughput RNA analysis technology. The genome encodes protein structure at two levels. In addition to the correspondence between RNA and protein primary sequences, a correlation exists between high levels of RNA structure and sequences that encode inter-domain loops in HIV proteins. This correlation suggests that RNA structure modulates ribosome elongation to promote native protein folding. Some simple genome elements previously shown to be important, including the ribosomal *gag-pol* frameshift stem-loop, are components of larger RNA motifs. We also identify organizational principles for unstructured RNA regions, including splice site acceptors and hypervariable regions. These results emphasize that the HIV-1 genome and, potentially, many coding RNAs are punctuated by previously unrecognized regulatory motifs and that extensive RNA structure constitutes an important component of the genetic code.

Genomes of all single stranded RNA viruses contain internal structures fundamental to viral replication and host defence evasion. Important viral RNA structures include internal ribosome entry sites, packaging signals, pseudoknots, transfer RNA mimics, ribosomal frameshift motifs, and *cis* regulatory elements[1,2]. In the human immunodefi ciency virus (HIV), RNA structures activate transcription, initiate reverse transcription, facilitate genomic dimerization, direct HIV packaging, manipulate reading frames, regulate RNA nuclear export, signal polyadenylation, and interact with viral and host proteins[2 6]. These RNA regulatory motifs have been identified by focusing on the 5′ and 3′ untranslated regions plus a few internal sequences. Most potential regulatory structures within viral RNA genomes, including in ~85% of the HIV 1 genome, are uncharacterized. This raises the possibility that new categories of RNA structure mediated regulation remain to be identified.

The HIV 1 genome is primarily a coding RNA and contains nine open reading frames that produce 15 proteins[2,3]. The Gag poly protein precursor is proteolytically processed to generate the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins. The Gag Pol polyprotein contains protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes a 30 amino acid signal peptide (SP), gp120 and gp41. Other sequences encode auxiliary proteins (Fig. 1a, grey boxes). Inside virions, HIV genomic RNA is found as a non covalent dimer, is 5′ capped and 3′ polyadenylated, and is annealed to a host $tRNA^{Lys3}$ molecule[2]. Viral proteins, especially nucleocapsid, chaperone the folding of HIV RNA[7].

Whole-genome structure analysis

To develop an accurate view of RNA structure in the full length genome, we analysed authentic genomic RNA extracted from HIV 1 virions. Our gentle purification maintained both previously char acterized secondary structures and the few known RNA tertiary structures. For example, the host $tRNA^{Lys3}$ was bound to the genome[2] and a pseudoknot in the 5′ untranslated region (UTR)[6,8] remained stably formed. The RNA was sufficiently intact to act as a template for primer extension reactions spanning the entire genome (Supplemen tary Table 1 and Methods).

High throughput selective 2′ hydroxyl acylation analysed by primer extension (SHAPE)[6,9 11] was used to chemically interrogate local nucleotide flexibility at 99.4% of the 9,173 nucleotides in the NL4 3 HIV 1 RNA genome. 1 methyl 7 nitroisatoic anhydride (1M7) preferentially acylates conformationally flexible nucleotides at the ribose 2′ OH position[9,10]. The resulting 2′ *O* adducts are detected as stops to primer extension using fluorescently labelled primers and capillary electrophoresis[6,10] (Fig. 3a) and are quantified by whole trace Gaussian integration[11] (Fig. 3b). SHAPE measure ments are reproducible between independent biological replicates (R^2 0.75; Supplementary Fig. 1). SHAPE reactivities are highly sensitive to local nucleotide flexibility and disorder, but are insens itive to solvent accessibility[9,12] (Supplementary Fig. 2).

SHAPE reactivities therefore provide direct model free informa tion about the overall level of structure, or architecture, for any RNA. The median SHAPE reactivity varies markedly across the HIV 1 genome (Fig. 1b, dark blue line). Regions with median reactivities below 0.25 indicate domains with substantial base paired secondary RNA structure, whereas median SHAPE reactivities of 0.5 and greater indicate regions of largely unstructured nucleotides.

We also assessed HIV 1 genome structure by examining evolu tionary information contained in nucleotide and amino acid vari ation to assign a pairing probability at each nucleotide[13,14]. This algorithm does not use chemical reactivity or thermodynamic information, and thus infers RNA structure using information that is orthogonal to SHAPE.

We identify at least 10 'structured' regions that exhibit both low SHAPE reactivity and high pairing probability (Fig. 1b, compare dark

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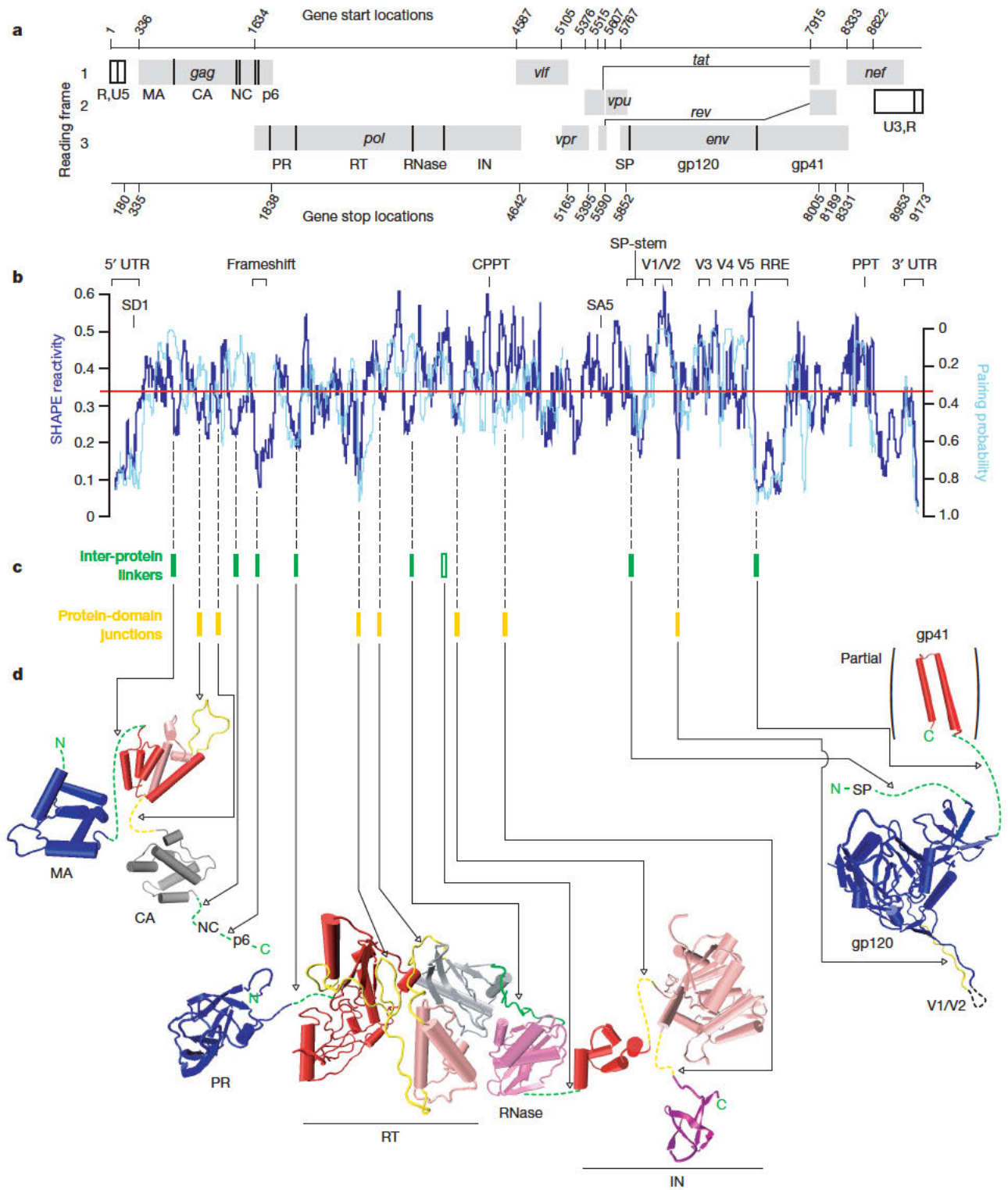

Figure 1 | Organization, extent of RNA structure, and relationship to protein structure for an HIV-1 genome. **a**, HIV 1 genome organization. Protein coding regions are shown as grey boxes; polyprotein domain junctions are depicted as solid vertical lines. Gene start and end sites are numbered according to NL4 3. CA, capsid; IN, integrase; MA, matrix; NC, nucleocapsid, PR, protease; RT, reverse transcriptase; SP, signal peptide. **b**, Comparison of median SHAPE reactivities (dark blue line) and evolutionary pairing probabilities (cyan line). Medians are calculated using a 75 nucleotide window. The global median (0.34) is depicted as a red line. Pairing probability is not reported for regions encoding overlapping reading frames. PPT, polypurine tract; CPPT, central PPT. **c**, Inter protein linkers in polyprotein precursors and the unstructured peptide loops that link protein domains are indicated with green and yellow bars, respectively. The single inter protein linker that is not encoded by a region of highly structured RNA (at the RNase H integrase junction) is shown with an open green bar. **d**, Comparison of domain structures for the large HIV proteins with the structure of the encoding RNA. Polyprotein linkers are green; inter domain loops are yellow; folded protein domains are blue, red, light magenta, purple and grey (Supplementary Table 2).

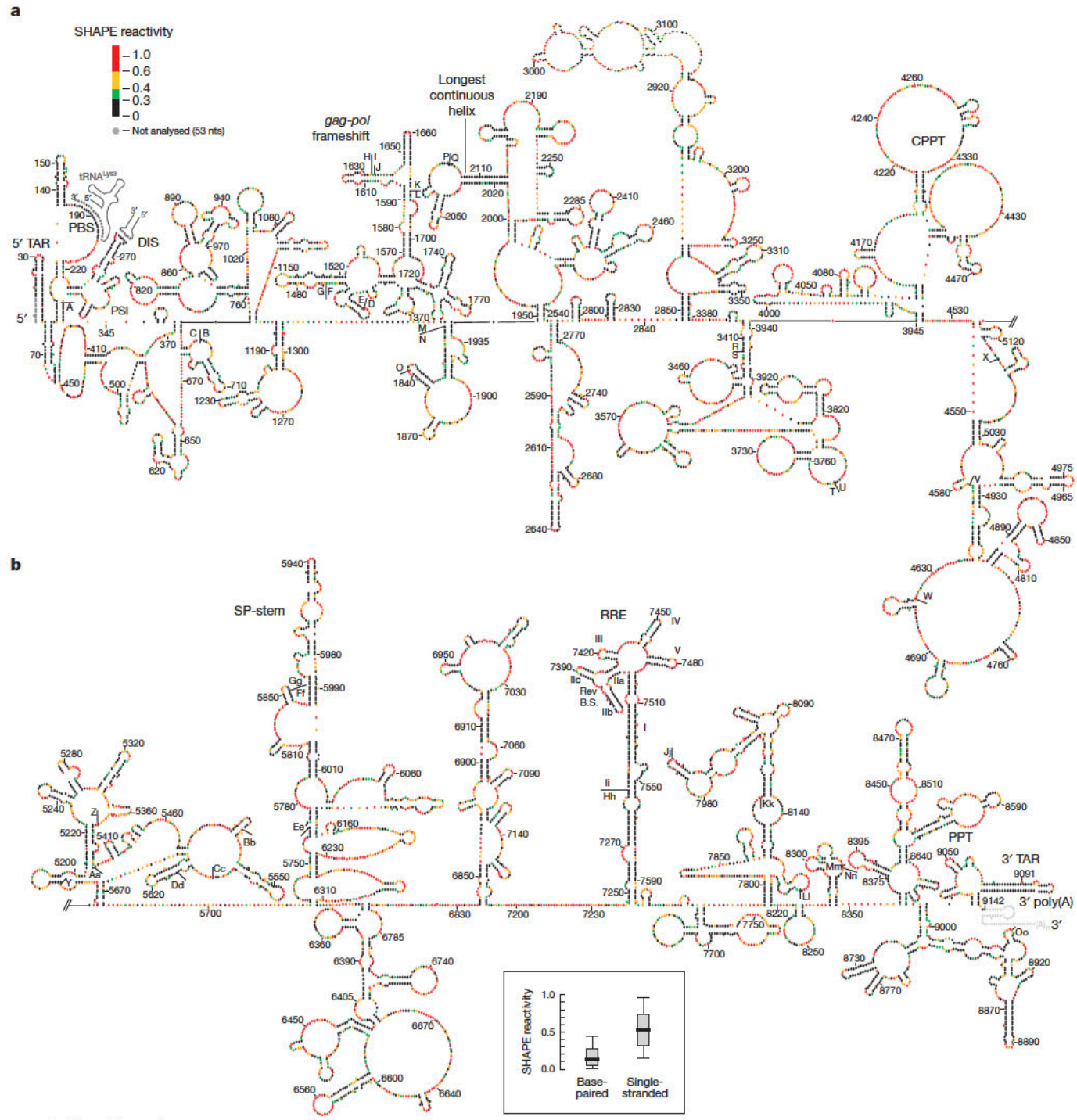

Figure 2 | Structure of the HIV-1 NL4-3 genome. The 5′ (**a**) and 3′ (**b**) genome halves are shown. Nucleotides are coloured by their absolute SHAPE reactivities (see scale in **a**). Every nucleotide is shown explicitly as a sphere; base pairing is indicated by adjacent parallel orientation of the spheres. Protein domains are identified by letters; TF, transframe peptide; nts, nucleotides. Important structural landmarks are labelled explicitly. Full nucleotide identities and pairings are provided in the Supplementary Information (Supplementary Fig. 7). Intermolecular base pairs involving the $tRNA^{Lys3}$ primer and the genomic dimer are shown in grey. Inset shows a box plot illustrating SHAPE reactivities for single stranded versus paired nucleotides in the model. Median reactivities are indicated by bold horizontal lines; the large box spans the central 50% of the reactivity data.

blue and cyan traces). This group includes the 5′ UTR and the Rev responsive element (RRE), which are known HIV regulatory elements (Fig. 1b). However, most of these highly structured and evolutionarily conserved elements have not been characterized previously. These regions include the protease reverse transcriptase junction, domains in the reverse transcriptase, integrase, and Vif open reading frames, an element 3′ of the Env signal peptide, and the *nef* 3′ UTR region.

We also identify at least seven 'unstructured' regions, extending over 200 600 nucleotides, with high SHAPE reactivities and low

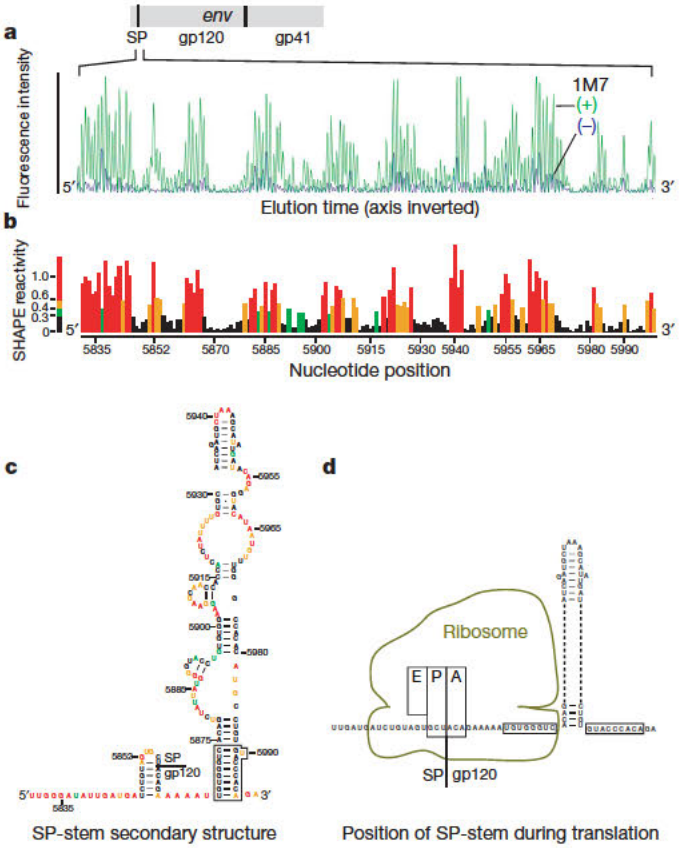

Figure 3 | SHAPE analysis of the signal peptide–gp120 region. a, Processed capillary electrophoresis trace showing intensity versus position for the (+) and (−) reagent reactions. **b,** Histogram of integrated and normalized SHAPE reactivities as a function of nucleotide position. The SHAPE reactivity scale shown here is used consistently throughout this work. **c,** RNA secondary structure model for the signal peptide pause site stem. **d,** Location of the signal peptide stem relative to the eukaryotic ribosome at the pause site. Base pairs disrupted when the ribosome is at the pause site are boxed.

pairing probabilities. These include the RNase H coding domain, variable domains (V*x*) in gp120, and the polypurine tract (Fig. 1b). On a smaller scale, the consensus sequences for the highly used splice site acceptors are also unstructured (Supplementary Fig. 3). There are four regions of apparent disagreement in the level of RNA structure, having high pairing probabilities and high SHAPE reactivities (one each in the reverse transcriptase, RNase, integrase and gp41 coding regions). This small group may reflect sequence conservation that is not accounted for by the evolutionary model[13], or may form critical structures at an alternative stage of the viral replication cycle.

RNA structure encodes protein structure

We first evaluated whether global RNA genome structure is linked to protein structure. HIV 1 produces three major classes of messenger RNA. The 9 kilobase (kb) class encodes Gag and Gag Pol and is identical to the packaged genomic RNA analysed here except, as an mRNA, it is not dimerized at its 5′ end[2]. There are very few differ ences in the SHAPE reactivity of dimeric and monomeric RNAs at the 5′ end of the genome[6]. Thus, genome structures outside of the dimerization region will correlate closely to the mRNA that encodes Gag and Gag Pol. The most abundant 4 kb *env* mRNA is generated by splicing nucleotide 288 (SD1, the major splice donor) to nucleotide 5522 (termed the SA5 site)[15]. SA5 is followed by an unstructured genome region (Fig. 1a, b). Thus, RNA structures identified in the *env* coding region probably exist in the spliced mRNA that encodes Env. Structures for the 1.8 kb class of mRNAs, which generate Tat and Rev, cannot be predicted using the genomic RNA because dis continuous segments are joined in the final mRNA.

The Gag, Gag Pol and Env polyprotein precursors are synthesized roughly as beads on a string, and the constituent proteins are liberated by proteolytic cleavage[2,3] (Fig. 1a, d). Eight inter protein peptides link the HIV proteins (Fig. 1c, green bars). The RNA sequences that encode these spacer peptide linkers in Gag (at the matrix capsid, capsid nucleocapsid and nucleocapsid p6 junctions), Pol (protease reverse transcriptase and reverse transcriptase RNase H junctions) and Env (signal peptide gp120 and gp120 gp41 junctions) all (except the RNase integrase junction) have SHAPE reactivities that are much lower than the median (Fig. 1b). RNA sequences that encode these inter protein peptide linkers are more highly structured than 95.2% of randomly selected regions in the genome (Supplementary Fig. 4a).

Domains in the individual HIV 1 proteins capsid, reverse tran scriptase and integrase are also linked by unstructured peptide ele ments, and each domain junction is encoded by an RNA region of low SHAPE reactivity (compare yellow bars in Fig. 1c with dark blue trace in Fig. 1b). Protein loops encoded by RNA regions with low SHAPE reactivity include the cyclophilin loop and the linker between the amino and carboxy terminal domains in capsid, both loops that link independently folded domains in reverse transcriptase, and the eight and nine amino acid loops linking the three domains in inte grase (Fig. 1d, in yellow). These protein domain junctions are more highly structured than 88.9% of randomly selected equivalent length regions in the genome (Supplementary Fig. 4b).

In contrast to the other large HIV proteins, domains in gp120 (termed inner, outer and bridging sheet) are not structurally auto nomous. The C terminal 35 residues of gp120 weave from the outer to the inner domain, and the bridging sheet is comprised of residues that are 315 positions distant[16]. Junctions between domains in gp120 are also not encoded by highly structured RNA, suggesting that gp120 folding is not linked to RNA structure in the same way as for other HIV proteins because its constituent domains are not structurally independent.

The recurring pattern of structure, conspicuously located near or after autonomously folding protein coding domains, is consistent with a model in which HIV protein structure is encoded in its RNA at two distinct levels. The first is the linear relationship between RNA and protein primary sequences. In the second level, higher order RNA structure directly encodes protein tertiary structure, because unstructured protein loops are derived from highly struc tured RNA elements. Many proteins appear to fold during trans lation[17], highly structured RNA slows and causes ribosomal pausing during translation[18,19], and changes in the extent of local RNA structure modulate protein activity[20]. Together, these observa tions suggest that attenuation of ribosome elongation by highly structured RNA at protein domain junctions facilitates native fold ing of HIV proteins by allowing time for domains to fold indepen dently during translation.

This model makes the clear prediction that ribosome pause sites should occur preferentially in the highly structured regions of an HIV 1 RNA that encode protein junctions. We tested this idea using a toeprinting experiment, in which ribosome processivity is inhibited by cycloheximide and sites preferentially occupied by the ribosome are detected as stops to primer extension in an *in vitro* translation reaction[21]. Ribosome pause sites are statistically overrepresented at the matrix capsid and capsid nucleocapsid junctions in Gag and at the sequences encoding the cyclophilin loop in capsid (Supplemen tary Fig. 5). Conversely, ribosome pause sites are underrepresented in flanking, but unstructured, regions of the HIV RNA (P 0.018). These experiments thus strongly support the model that mRNA structure over a region spanning 60 100 nucleotides specifically modulates ribosome processivity at protein domain junctions.

RNA secondary structure model for HIV-1

Comprehensive SHAPE reactivity information can also be used to determine a nucleotide resolution secondary structure model for the entire NL4 3 HIV 1 genome (Fig. 2). SHAPE reactivities are converted

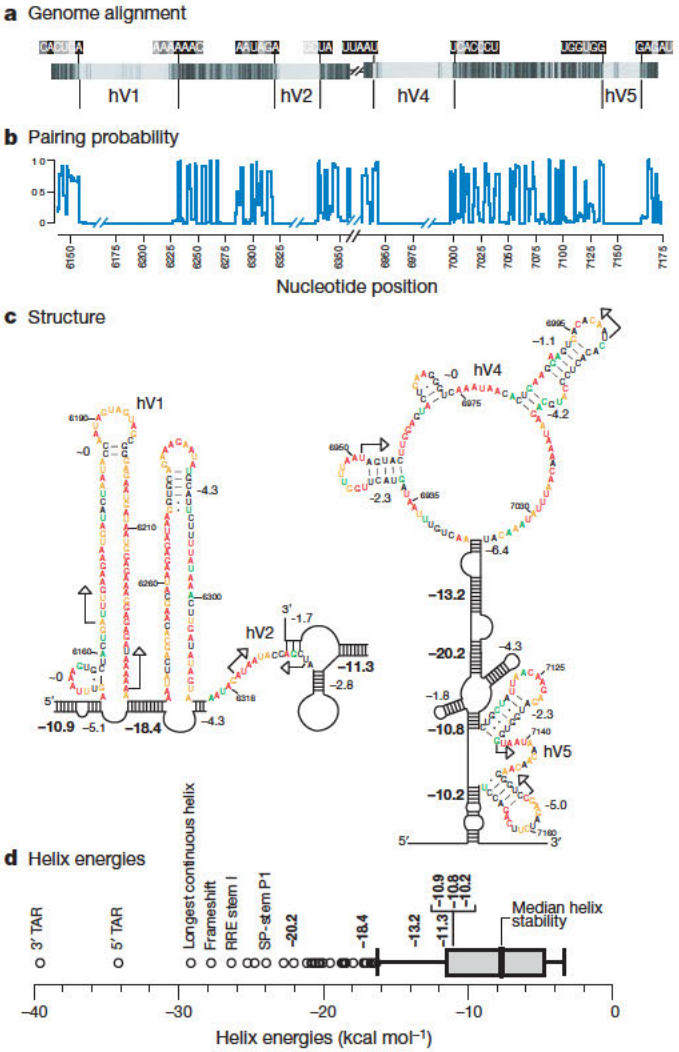

Figure 4 | RNA structure in Env hypervariable regions. **a**, Schematic sequence alignment for group M reference sequences[14] at the Env hypervariable regions (hV1, hV2, hV4 and hV5). Nucleotides are represented as vertical bars; light grey and black indicate low versus universal conservation, respectively. **b**, Evolutionary pairing probabilities. Breaks indicate extensive nucleotide insertions and deletions among the group M consensus sequences. **c**, RNA structures at the hypervariable coding regions hV1, hV2, hV4 and hV5. Calculated free energies are shown for each helix (in kcal mol ^{1}); energies for anchoring helices proposed to function as structural insulators are emphasized in bold. **d**, Distribution of helix stabilities in the HIV genome shown in a box plot representation. Whiskers illustrate 1.5 times the interquartile range, and circles emphasize helices of exceptionally high stability. Free energy changes for proposed insulating helices are in bold; other significant helices are labelled.

to free energy change terms and used to constrain a thermodynamic folding algorithm[22,23]. The final result is a thermodynamically favoured structural model highly reflective of the experimental SHAPE data, at single nucleotide resolution. For example, most nucleotides assigned to single stranded regions are reactive towards SHAPE (Fig. 2, red, orange and green nucleotides), whereas base paired nucleotides are predominantly unreactive (Fig. 2, black nucleotides and inset). For a full discussion of SHAPE directed RNA folding and the fundamental correctness of this model, see the Methods.

The HIV 1 genome is less structured than ribosomal RNA but, similarly, contains independent RNA folding domains that extend from the overall genomic backbone. These domains include both small stem loops plus roughly 21 large and complexly folded structures (Fig. 2). Although many genome regions are highly structured, only seven helices span a complete turn of an 11 base pair (bp) RNA duplex. The largest paired region, devoid of bulges, is the structured RNA element that bridges the coding junction between the reverse transcriptase and RNase H folding domains (Fig. 1). This helix is 19 bp long, contains a non canonical G A base pair (Fig. 2a, nucleotides 2015 2033/2103 2121), and is thus shorter than the 30 bp length competent to induce the interferon response[24].

The HIV 1 genome structural model provides a robust starting point for identifying previously unrecognized functional elements and long range RNA interactions. SHAPE reactivities describe a well formed stem 3′ to the signal peptide coding region in the Env protein (Fig. 3c). This stem (the signal peptide stem) is evolutionarily conserved (Fig. 1b), reinforcing an important biological role. The signal recognition particle (SRP) binds the nascent Env signal peptide and translocates the cytoplasmic ribosome elongation complex to the rough endoplasmic reticulum, where translation of gp120 and gp41 continue[25].

RNA induced translational pausing occurs as the ribosome unwinds highly structured RNA, typically located 6 7 nucleotides downstream of the A site[18]. The signal peptide stem will be exactly in this conformation when the final $tRNA^{Ala}$ from the signal peptide and the first $tRNA^{Thr}$ of gp120 are in the P and A sites (Fig. 3d, boxed nucleotides). We infer that ribosomal attenuation or pausing at the signal peptide stem provides more time for SRP recruitment and subsequent translocation of the elongation complex to the endoplasmic reticulum.

The SHAPE constrained secondary structure is also informative for previously identified regulatory motifs. In HIV 1, *pro* and *pol* gene products are translated when the ribosome undergoes a 1 register shift from the *gag* to the *pol* reading frames. Frameshifting occurs at a slippery sequence (UUUUUUA) and is enhanced by a downstream RNA structure. These elements are typically drawn as a single stranded slippery sequence and a 12 bp stem loop[26]. Direct analysis of intact genomic RNA shows that the *gag pol* frameshift signal is one component (identified here as P3) of a three helix structure (Fig. 2 and Supplementary Fig. 6a). The slippery sequence pairs to form one of the three helices (P2). These two helices are stabilized by an anchoring helix (P1) that creates this discrete structural element (Supplementary Fig. 6a). This three helix junction structure is conserved among HIV 1 group M sequences (Supplementary Fig. 6b).

Most RNA viruses require a complex pseudoknotted structure to induce ribosomal frameshifting[27]. The three helix junction may function, in part, to slow translation before the ribosome encounters P3, facilitating the prerequisite pause necessary for frameshifting. The three helix junction model may also explain why changing the slippery site to sequences that allow alternative tRNA pairing and enhance frameshifting in other RNA viruses eliminates frameshifting in HIV 1 (ref. 28). In the SHAPE directed model, changes to the slippery sequence compromise base pairing in the conserved P2 helix (Supplementary Fig. 6).

Unstructured motifs and insulator helices

Analysis of the HIV 1 genome structure supports a role for RNA structures in sequestering unstructured regions. Five variable domains (V1 V5; see Fig. 1a, b) in the Env surface protein, gp120, account for much of the genetic diversity in HIV 1 (ref. 14) and are a critical component of the viral host evasion strategy. Four of these domains are hypervariable (hV1, hV2, hV4 and hV5) and exhibit large amino acid insertions and deletions between viral isolates[14].

Sequences encoding hypervariable domains are internally unstructured and are bordered by evolutionarily conserved and stable RNA structures (Fig. 4a, b). For example, hypervariable region hV1 is encoded by RNA sequences with high SHAPE reactivities and is flanked by two stable helices (with free energies of 10.9 and 18.4 kcal mol ^{1}, Fig. 4c). Similar patterns are evident in the other hypervariable regions (Fig. 4c). Some hypervariable regions, especially hV4, contain internal helices with non trivial free energies; however, these helices are not evolutionarily conserved (Fig. 4b)

and are much less stable than the flanking helices that have stabilities in the 10 20 kcal mol ^{1} range (Fig. 4c). These helices are also highly stable relative to the distribution of duplex stabilities over the entire genome (Fig. 4d).

Collectively, these data suggest that RNA sequences encoding length polymorphisms in *env* are segregated from the rest of the genome by stable helices that function as structural insulators. The observed organization of hypervariable regions is thus well suited, first, to accommodate extensive substitutions, insertions or deletions, and second, to prevent these regions from forming non functional base pairing interactions with adjacent regulatory motifs, which include the 3′ splice site acceptors and the RRE.

Perspective

Structural analysis of a complete HIV 1 genome reveals that this RNA is punctuated by previously unrecognized, but readily identifi able and evolutionarily conserved, RNA structures. Most genome regions with low SHAPE reactivities are associated with a regulatory function (Fig. 1). SHAPE may be generally useful for identifying new regulatory elements in large RNAs. All of these elements represent hypotheses and starting points that we hope will stimulate further detailed examination. Our discovery that the peptide loops that link independently folded protein domains are encoded by highly struc tured RNA indicates that these and probably other mRNAs encode protein structure at a second level beyond specifying the amino acid sequence. In this view, higher order RNA structure directly encodes protein structure, especially at domain junctions. The extraordinary density of information encoded in the structure of large RNA mole cules (Figs 1, 2 and 4d) represents another level of the genetic code, one which we understand the least at present. This work makes clear that there is much to be discovered by broad structural analyses of RNA genomes and intact mRNAs.

METHODS SUMMARY

Full length HIV 1 genomic RNA was gently purified from NL4 3 virions (GenBank accession AF324493). The RNA was equilibrated in a native buffer (50 mM HEPES (pH 8.0), 200 mM potassium acetate (pH 8.0), 3 mM $MgCl_2$) at 37 °C for 15 min and treated with 1M7 (ref. 10). Sites of 2′ hydroxyl modifica tion were identified over read lengths spanning several hundred nucleotides using 31 primer extension reactions resolved by fluorescence detected capillary electro phoresis[6,11]. Pairing probabilities were determined using RNA Decoder[13] and secondary structure models were developed by incorporating SHAPE reactivities as a pseudo free energy change term, in conjunction with nearest neighbour parameters, in an accurate thermodynamics based prediction algorithm[22,23].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.M.W., R.J.G. and K.M.W. conceived of and designed the HIV 1 genome structure analysis project. J.M.W. and K.M.W. analysed and interpreted the HIV SHAPE structure information. K.K.D., R.S. and C.L.B. designed and performed the bioinformatic pairing probability analysis. J.M.W., R.J.G. and C.W.L. performed the experiments. J.M.W., C.L.B. and K.M.W. performed the statistical analyses. J.W.B. produced and purified HIV 1 virions. J.M.W. and K.M.W. wrote the manuscript with contributions from all authors.

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METHODS

Virus production. HIV 1 strain NL4 3 (group M, subtype B) was used to infect a non Hodgkin's T cell lymphoma cell line (a modified version of the SupT1 cell line, which was a gift from J. Hoxie)[29]. The virus containing inoculum for infecting SupT1 cells was generated by $CaPO_4$/DNA coprecipitation[30] and sub sequent transfection of pNL43 (NIH AIDS Research and Reference Reagent Program; GenBank accession AF324493) into 293T cells[31]. HIV 1 virions were purified as described[32] except cells were removed using a Millipore Opticap XL 5.0 micron filter. The total protein and CAp24 yields were 20.7 mg and 2.3 mg, on the basis of total protein (BioRad DC protein assay) and HPLC with sub sequent amino acid analysis assays, respectively.

Virions were purified from cellular debris by subtilisin treatment and cent rifugation through a sucrose cushion. Concentrated virions (in 19 ml, corres ponding to 19 l of infected cell culture supernatant) were digested with subtilisin (1 mg ml $^{-1}$, in 20 mM Tris (pH 8.0), 1 mM $CaCl_2$, 37 °C, 18 h; stopped by the addition of 5 μg ml $^{-1}$ phenylmethylsulphonyl fluoride[33]). The resulting solution contained digested cellular proteins and viral particles free of surface proteins. The sample was centrifuged through a cushion of 20% (w/v) sucrose in PBS (Beckman SW41 rotor, 235,000*g*, 2 h, 4 °C); supernatant was carefully removed, and residual sucrose in the pellet was removed by overlaying PBS and repeating the centrifugation step (1 h at 4 °C).

RNA extraction. The key features of this protocol are that genomic RNA was gently extracted from purified virions in the presence of buffers containing monovalent and divalent ions, consistent with maintaining RNA secondary and tertiary structure. The HIV genomic RNA was not denatured by heat, chem ical denaturants, magnesium chelation, or removal of monovalent cations dur ing this process. Subtilisin treated virions were suspended in virion lysis buffer (VLB; 50 mM HEPES (pH 8.0), 200 mM NaCl, and 3 mM $MgCl_2$) and lysed with 1% (w/v) SDS and 100 μg ml $^{-1}$ proteinase K (~22 °C, 30 min). The digest was extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1, pre equilibrated with VLB), followed by two extractions with pure chloroform. Quantitative reverse transcriptase PCR was used to quantify viral RNA yields against a standard curve[34–36]. The total yield from 19 l of infected cells was 97.2 pmol HIV 1 genomic RNA. The aqueous layer (3.6 ml) was brought to 300 mM NaCl and precipitated with 70% (v/v) ethanol. Retroviral genomes commonly contain single stranded breaks[2]. Approximately 30% of our genomic RNA was intact, as judged by visualization in agarose/formaldehyde gels; nicks in the remaining 70% seemed to be roughly randomly distributed on the basis of direct visualization of the genomic RNA and from the continuity of our primer extension reactions (see Supplementary Table 1).

RNA modification. The RNA pellet containing 97.2 pmols of HIV 1 genomic RNA was dissolved in 880 μl of modification buffer (50 mM HEPES (pH 8.0), 200 mM potassium acetate (pH 8.0), 3 mM $MgCl_2$) and incubated at 37 °C for 15 min. Then, 405 μl of the solution was added to 45 μl pre warmed (37 °C) 1M7 (in dimethylsulphoxide (DMSO))[10] or to DMSO. After 4 min, 45 μl of 50 mM EDTA (pH 8.0) were added to each tube. The reactions were divided into 11 μl aliquots and precipitated with ethanol.

Primer synthesis. Primers were designed with the aid of OligoWalk, part of the RNAstructure software package[22] (available for download at http://rna.urmc. rochester.edu/) (Supplementary Table 1). Primers were required to be 20 22 nucleotides in length, have high melting temperatures and low self annealing energies, and preferably end with a 3′ G or C. Only 2 out of 31 primers required redesign, giving OligoWalk a 94% success rate. Primers were synthesized to contain a 5′ six carbon linker terminating in a primary amine (IDT). The amine tethered DNA primers (1 μl; 25 μg ml $^{-1}$) were labelled with one of four fluorophores (5 FAM, 6 JOE, 6 TAMARA or 5 ROX; AnaSpec) using *N* hydroxysuccinimide chemistry (3 μl NHS coupled dye (20 mg ml $^{-1}$ in DMSO) in 0.1 M $NaBO_3$ HCl (pH 8.5); ~22 °C, 3 h). Labelled primers were precipitated with ethanol, purified on a denaturing gel (20% 29:1 acryalmide/ bis acrylamide, 7 M urea, 1× TBE), recovered by passive elution in water, pre cipitated (300 mM NaCl, 2.5 vol ethanol, 1 vol isopropanol), pelleted, and dis solved in water. Spectrophotometric measurements indicated labelling was ~90 95% efficient as determined by the [dye]/[DNA] ratio.

Primer extension. RNA pellets (1 pmol) were dissolved in 10 μl 0.5× TE (5 mM Tris (pH 8.0), 0.5 mM EDTA) and mixed with 3.0 μl of 0.4 μM primer. The (+) and (−) 1M7 reagent reactions were labelled with JOE and FAM, respectively. Primers were annealed to the RNA by heating to 65 °C for 5 min and 45 °C for 2 min, and then placed on ice. Six microlitres of reverse transcriptase mix[37] (SuperScript III, 5× buffer, DTT, dNTPs; Invitrogen) was added to each tube and incubated for 10 s at 45 °C, 5 min at 52 °C, 5 min at 65 °C, and cooled to 4 °C. Sodium acetate (pH 5.2; 2.0 μl at 3 M) was added to each tube, (+) and (−) 1M7 tubes were combined, and 120 μl of ethanol was added to precipitate the cDNA products. The reactions were pelleted, washed with 70% ethanol, and dissolved in 10 μl deionized formamide.

Sequencing. Dideoxy sequencing reactions (GenomeLab Methods Development Kit; Beckman) were performed using plasmids pDR0 and pDR25 (containing partial NL4 3 sequences), and primers were labelled with TAMARA and ROX. Primer sequences were identical to those in Supplementary Table 1 except primer 31, the sequence (5′ CTGCAACCTCTACCTCCTG GGTGCTAGAG 3′) of which annealed to the plasmid rather than the poly(A) RNA sequence in the genomic RNA.

Capillary electrophoresis. cDNA fragments were resolved by capillary electro phoresis[6,10] (Applied Biosystems AB3130 instrument). Samples were injected at 1.2 kV for 16 s into a 36 cm capillary containing POP7 (ABI) and subjected to electrophoresis for 25 min at 15 kV. The fluorescence detector was initially calibrated with 5 FAM, 6 JOE, 6 TAMARA and 5 ROX using fluorescent markers with fragment lengths of 242 (5 FAM), 206 (6 JOE), 188 (6 TAMARA) and 155 (5 ROX) nucleotides. Fragments were generated by linear amplification of HindIII digested plasmid pUC18 using primers with the sequences 5′ CAGAGCAGATTGTACTGAGAG 3′, 5′ GTGAAATACCGCAC AGATGC 3′, 5′ GCGTAAGGAGAAAATACCGCATC 3′ and 5′ CGCCATTC AGGCTGCGCAACTG 3′, labelled with 5 FAM, 6 JOE, 6 TAMARA and 5 ROX, respectively. Fluorescent spectral overlap based on this DNA ladder was calibrated using AB3130 software.

Data processing. Raw electropherograms, containing fluorescence intensity ver sus elution time information, were converted to normalized SHAPE reactivities using ShapeFinder[6,11,23] (available for download at http://bioinfo.unc.edu). The ShapeFinder software aligns the (+) and (−) reagent traces to the two dideoxy nucleotide sequencing ladders, corrects for signal decay[38], and performs a whole channel Gaussian integration[11] to quantify all individual peak areas (see Fig. 3a). Only 11 of the 9,173 nucleotides in the NL4 3 RNA genome had high back ground and were therefore excluded from analysis. Data sets were normalized to a scale such that 1.0 represents the average intensity of highly reactive nucleotide positions[6,23]. On this scale, ~95% of integrated intensities for the HIV 1 genome fall between 0 and 1 (see histogram in Fig. 3b). Each primer extension reaction was processed individually. The resulting intensities in regions with overlapping data from different primers correlated closely: reactivity differences were typ ically less than 0.1 SHAPE unit. Regions with overlapping data accounted for ~25% of the total nucleotide positions and were averaged to generate the final data set spanning the entire NL4 3 genome.

Toeprinting ribosome pause sites at the matrix capsid and capsid nucleocapsid junctions. A double stranded DNA template to direct synthesis of an mRNA spanning NL4 3 Gag nucleotides 1 to 1795 was generated by PCR. This region encompasses the entire 5′ UTR and most of the *gag* coding region and ends after the three stem frameshift element. The 5′ primer included a T7 promoter sequence (5′ TAATACGACTCACTAATGGTCTCTCTGGTTAGACCA 3′), and the 3′ primer (5′ GCTAAAGGTTACAGTTCCTTGTC 3′) encoded a stop codon at position 1787. The RNA transcript was capped and polyadenylated (mSCRIPT, Epicentre) and *in vitro* translation was carried out in rabbit reticulocyte extract (Ambion) using ~60 μg of the capped, polyadenylated transcript, 1 μl 1.25 mM L methionine, 1 μl ^{35}S methionine (PerkinElmer), 17 μl reticulocyte extract, and 1.25 μl 20× 'medium salt' translation buffer (Ambion) in a total volume of 26 μl at 37 °C. Cycloheximide was added at 0, 7, 15 or 45 min to arrest translation[21]. Translation reaction aliquots were separated on an 8 16% SDS PAGE gel (Invitrogen) to confirm production of a protein of the correct length. Sites of ribosome pausing were detected by adding the following to 25 μl of the *in vitro* translation mixture: 1.35 μl 10 mM each dNTP, 2 μl 4.0 μM fluorescently labelled primer (primer 4 or 6 for interrogating the matrix capsid and capsid nucleocapsid regions, respectively), 1 μl 200 mM $MgCl_2$, and 2 μl Superscript III (Invitrogen). The translation reaction that was pre quenched with cycloheximide was taken as background and was resolved using a JOE labelled primer. The 7, 15 and 45 min time points were resolved using FAM labelled primers. Primer extension reactions were incubated at 37 °C for 30 min and stopped by the addition of 1 μl 0.5 M EDTA and 400 μl water. The reaction was extracted with phenol:chloroform:isoamyl alco hol (25:24:1, 2×) and chloroform (1×). Four microlitres of this solution, 1 μl of a cDNA sequencing ladder, and 15 μl of formamide were combined, heated to 105 °C for 5 min, and resolved by capillary electrophoresis. Toeprinting traces were pro cessed with ShapeFinder[11] and normalized to a scale in which 1.0 is equal to the mean intensity of the most reactive positions, identically as described above for SHAPE experiments.

RNA secondary structure model. The entire NL4 3 sequence 9,173 nucleo tides plus 20 3′ adenosines (representing the poly(A) tail) was folded using the thermodynamics based algorithm in RNAstructure[22,23]. SHAPE information was used to constrain secondary structure calculations by incorporating SHAPE reactivities as pseudo free energy change terms[6,23] using slope and inter cept values of 30 and −6, respectively. The maximum distance allowed between any two paired positions was 600 nucleotides. The slope and intercept values are derived from previous parameterization on long RNAs, and the 600 nucleotide

cutoff reflects that 99% of all base pairs in ribosomal RNA occur between nucleotides less than this distance apart[23]. The genome was initially folded as a single (9,193 nucleotides) unit; folding was then fine tuned by dividing the RNA into five independent folding regions, separated by long stretches of reactive nucleotides that were calculated to be unpaired when the entire genome was folded with SHAPE constraints (NL4 3 residues 1 2844, 2836 5722, 5676 6832, 6807 7791 and 7779 9193). Dividing the genome in this way facilitated model building and prevented the formation of a few poorly supported long distance pairings between domains. Highly reactive nucleotides at the termini of each region were prohibited from forming base pairs in these region specific calcula tions. Helices consisting of a single base pair were removed from the final model and unreactive nucleotides in the primer binding site (183 199) were taken to reflect hybridization with the tRNA primer. The current version of our algorithm does not allow pseudoknots and therefore our HIV 1 structure model (Fig. 2) includes only one, heuristically predicted[6,8], pseudoknot.

Quality of SHAPE directed model of the entire HIV 1 genome. The algorithm by which SHAPE information is used to create an RNA secondary structure model does not make any specific assumptions about the magnitude of SHAPE reactivity that corresponds to single stranded versus based paired regions. Instead, SHAPE reactivities are converted to free energy change terms and used to constrain a thermodynamic folding algorithm[22,23]. SHAPE information is essential for generating this secondary structure model. Folding the genome by free energy minimization alone, using a best of class algorithm[22,39], results in a structure that is very different from the experimentally supported model. Only 47% of the base pairs in the SHAPE directed model also occur in the lowest free energy thermodynamics only model. The unconstrained thermodynamics only model is readily shown to be incorrect because many regions with high SHAPE re activities are assigned as paired in the unconstrained model, and many regions with low SHAPE reactivities are assigned as single stranded.

Several lines of evidence support fundamental correctness of our working SHAPE directed HIV 1 genome structural model (Fig. 2). First, SHAPE directed folding is well validated and predicts the known structures of large RNAs, includ ing 16S ribosomal RNA, with high accuracies (>90%)[10,23]. Second, most nucleo tides assigned to single stranded regions are reactive by SHAPE (Fig. 2, red, orange, and green nucleotides). Conversely, base paired nucleotides are generally unreactive (Fig. 2, black nucleotides and inset). Thus, the structural modelling faithfully incorporates the experimental data. Third, many single nucleotide bulges are predicted as single reactive positions imbedded in helices with flanking nucleotides that are unreactive towards SHAPE, which speaks to the accuracy at the single nucleotide resolution level (for select examples see Fig. 2, positions 1725, 3376, 4891, 5990, 7431, 7568 and 9091). Fourth, previously characterized HIV RNA structures including the 5′ TAR element, the DIS component of the packaging signal, and the five stem RRE, serve as positive controls and form structures consistent with previous work[4,40] (Fig. 2). In the case of the *gag pol* frameshift structure, we note that SHAPE data do not support common alterna tive proposals for this specific structure, including either a longer bulged stem or a pseudoknot.

Most structures in our current HIV 1 genome model, especially in regions with several closely spaced helices, are extremely well determined, as evidenced by the strong correlation between SHAPE values and base pairing. This correlation is also consistent with benchmarking studies showing that SHAPE reactivities strongly discriminate between base paired and single stranded nucleotides (Supplementary Fig. 2)[41] and are proportional to the extent of local nucleotide disorder[12]. In contrast, some of the larger loop regions in our model may reflect regions that interconvert between multiple structures[38,42]. Elements that may require future refinement include the precise termini of helices at some multi helix junctions and along the central backbone of the genome structure and the identification of further pseudoknot and long range interactions.

Calculation of evolutionary base pairing probabilities. RNA Decoder[13] was used to identify regions in the HIV 1 genome in which the ability to form base pairs is evolutionarily conserved. The program takes a set of grammar parameters, a multiple sequence alignment, and a phylogenetic tree as input. The output is a pairing probability for each position in the genome, given the phylogenetic tree, alignment, and the grammar structural model. The pairing probability for posi tion i in alignment D is the sum over all stem structural labels (k) of $P(\pi_i = k|M)P(D|\pi,T,M)$, where π is the structure, M is the grammar model parameters, and $P(\pi_i = k|M)$is the posterior probability that position i has the specific structural label k, given the grammar[43], and is calculated by the inside outside algorithm[44]. In Bayesian terms, $P(\pi_i = k|M)$ is the prior probability of structure π and $P(D|\pi,T,M)$ is the alignment probability, calculated using the Felsenstein algorithm[45]. Pairing predictions were made using an alignment of non recombinant group M subtype reference sequences obtained from the Los Alamos HIV database[14], with minor manual editing (and excluding subtype G, which is now considered a circulating recombinant form[46]). Codon positions in genome regions encoding more than one protein in overlapping reading frames were defined according to the first open reading frame in the following pairs: *gag pro*, *pol vif*, *vpr vif*, *vpr tat*, *rev tat*, *env vpu*, *env tat2* and *env rev2*. Owing to differences in nucleotide content and evolution rate within different genes in the HIV genome, the genome was scanned in two sections, upstream and down stream, that overlapped in the *vif* gene. This allowed use of separate phylogenetic trees for each scan, with branch lengths calculated according to the rates of evolution in each genome region. The phylogenetic tree for the 5′ half was built using the third codon position for the *gag*, *pol* and *vif* genes, and the 5′ non coding region; the tree for the 3′ half was built on the third positions of *vif*, *vpr*, *rev*, *vpu*, *env* and *nef* genes, and the 3′ non coding region.

Pairing probabilities were assessed across the entire genome. To accommodate as many pairing interactions as possible, we used a large window size (1,300 nucleotides), and spaced the scans at 300 nucleotide intervals. Pairing probabilities for each scan were combined using the statistical program R[47] taking the maximum pairing probability in overlapping windows. It is important to note that high pairing probabilities identify regions experiencing evolutionary pressure to retain a specific, defined, secondary structure. A low pairing probability, although sug gestive of a lack of structure, can also reflect (1) that an additional evolutionary constraint exists that is not accounted for by the evolutionary model, or (2) that natural selection favours folding in general, but not a precise pattern of folding.

Bootstrap analysis of SHAPE reactivities in inter protein linkers and protein domain junction. A bootstrap procedure was used to compare the SHAPE re activities of particular collections of genome elements to the expectation for random genome regions of the same size. For a comparison to a collection of n genome elements, we generated 100,000 bootstrapped samples by randomly choosing n locations from the relevant portion of the genome, and randomly assigning the lengths of the actual genome elements to these n locations. For comparison to the protein domain junctions, locations were drawn randomly from the entire coding portion of the genome (bases 336 8621). We specified a length of 60 nucleotides for each region. For comparison to the intra domain loops, locations were drawn randomly from within the domains where loops occur and assigned lengths that reflected loop sizes in the same domain (for example, for the capsid domain, one element of 45 base pairs was drawn from within bases 732 1427). Bootstrap samples that contained overlapping genome regions were thrown out. The mean SHAPE reactivities for each bootstrap sample were used to generate a frequency distribution that describes the expectation for equally sized but randomly located collections of genome elements in HIV coding regions. We obtained a P value by determining the percentage of the bootstrapped means that was lower than the mean SHAPE reactivity for the collection of genome elements. This P value is equivalent to the probability that the low SHAPE reactivity in the actual collection of genome elements occurred by chance. P values for inter protein linkers and protein domain junctions were 0.0482 and 0.0777, respectively. The reverse transcriptase RNase H junction functions both as an inter protein linker and as a protein domain junction because it is cleaved one half of the time. For this analysis, the reverse transcriptase RNase H junction was counted as an inter protein linker.

Statistical analysis of ribosome pause sites. Toeprinting data spanned 748 nucleotides (positions 670 1018 and 1243 1652; Supplementary Fig. 5). In these two reads, there were 220 nucleotides that fell within 30 nucleotides of the matrix capsid, capsid nucleocapsid, or nucleocapsid p6 junctions or in the cyclophilin loop. We evaluated whether ribosomes pause preferentially near protein junctions using the binomial distribution. A total of 36 base pairs yielded toeprint signals with an intensity of 1.0 or greater. A signal of 1.0 corresponds approximately to 1.5 standard deviations above the mean; 17 of these occurred within 30 nucleotides of a protein junction. The probability of observing this distribution by chance is P 0.018. This analysis was insensitive to the choice of high signal threshold. Similar P values were obtained for toeprint thresholds between 0.6 and 1.6.

Consensus structure. The *gag pro pol* consensus structure (Supplementary Fig. 6b) was generated by aligning the 37 reference group M HIV 1 sequences[14] using CLUSTALW[48]. Regions of covariation were identified using a sequence logo[49].

Helix energies. Helix free energy changes (Fig. 4c, d) were calculated using the RNAstructure program[22] as the sum of the base pair stacking nearest neighbour parameters[50,51]. Duplex regions containing single nucleotide bulges were taken to be a single helix. The helix free energy changes do not include penalties for terminal AU or GU pairs because these are, by convention in RNAstructure, associated with the loop formation free energy changes.

RNA and protein structure display. RNA secondary structures were composed using xrna (http://rna.ucsc.edu/rnacenter/xrna); HIV protein images (Fig. 1d) were created using Visual Molecular Dynamics[52].

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